INVESTIGATION OF *PHYTOPHTHORA* COMPONENTS INVOLVED IN PLANT PATHOGENESIS

BY ANDREA V. ROBOLD

A THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
OF
THE AUSTRALIAN NATIONAL UNIVERSITY

NOVEMBER 2003

Dedication

I dedicate this thesis to three members of my family:

My father, Otto Joseph Robold, who died during the course of this thesis,

My partner, Thomas Joseph Magill, who I married during the course of this thesis,

And our son, Martin John Magill, who was born during the course of this thesis.

STATEMENT

All research reported in this thesis is original and my own
except where acknowledgement is given,
and has not been submitted
for any other degree.

Andrea V. Robold

Acknowledgements

This thesis could not have been finished without the support of my family and friends. I would like to thank all the people involved.

I would like to thank my supervisor, Prof. A.R. Hardham for her encouragement, enthusiasm, and support throughout the course of my research. I know that especially the final months of writing this opus I could not have done without her.

I would like to thank my advisors, Dr. Jerry Marshall, Dr. Weixing Shan, and Dr. David Jones, for their invaluable suggestions. I would especially like to thank Jerry for his input even after his retirement.

Our technical officers, Jan Elliot and Val MacLean, thank you for endless advice. Jan, thanks to you the antibody fusion went extremely well and made it possible for me to clone an important gene. I hope your ankles will support many more Volleyball games. Val, I really hope you will enjoy your retirement.

Thanks to everyone else in the lab - Leila, Duka, Miki, and Reena – you always gave advice when needed and also listened to me whinge when nothing worked.

Thanks to the EM unit, especially Sally and Lily who never got tired of trying to explain the mysteries of transmission electron microscopes to me.

Thanks to all the other people here at Plant Cell Biology. You always made me feel welcome and I must say it is a pleasure to work with so many friendly, supportive, and helpful people.

Special thanks to my friends, Madeleine Rashbrook, Corinna Paeper, Dr. Katharina Siebke, Dr. Regina Himmelspach, Megan Hemming, and Ryan Wilson. You were always there for me in times of misery (and there were plenty of these)! Madeleine, I know my wedding dress would never have been finished in time if it were not for your expert skills in sewing.

Finally, I would like to thank my family, especially you, Thomas, for minding Martin, and your never-ending patience and love. And for your expertise with Photoshop! And for the cooking.

Abstract

The main aim of the present study was the molecular characterisation of components in zoospores of *Phytophthora* species that are likely to play important roles in the pathogen dissemination and the establishment of disease. The study focused on components associated with zoospore motility and adhesion.

In the first part of this study, a *Phytophthora nicotianae* zoospore cDNA expression library was screened using a monoclonal antibody directed towards the shaft of tripartite tubular hairs present on the anterior flagellum of *Phytophthora* zoospores. These hairs are responsible for forward movement of the spores. A positive cDNA clone was purified and sequenced. Data analysis showed that two unlinked inserts were present in the cDNA clone, both of which encoded putative protein kinases.

In the second part of this study, monoclonal antibodies directed towards vesicles within zoospores of the oomycete *Lagenidium giganteum* were screened against *Phytophthora cinnamomi* zoospores in immunofluorescence assays and in immunoblots of crude zoospore protein extracts. Thirteen of the *Lagenidium*-directed antibodies gave rise to small fluorescent spots in *P. cinnamomi* zoospores and to strong labelling of the surface of cysts. However, none of these antibodies recognised components in the ventral, dorsal or large peripheral vesicles in the *Phytophthora* zoospores.

In the third part of this study, new monoclonal antibodies were generated towards a microsomal preparation from *P. nicotianae* zoospores. Hybridoma supernatants were screened using immunofluorescence microscopy and immunoblotting, and 40 monoclonal antibodies that reacted strongly with the *P. nicotianae* spores were identified. At least eight different antigens were targeted within the zoospores. The occurrence of the antigens labelled by these antibodies was investigated throughout the asexual life cycle of *P. nicotianae* with the exception of chlamydospores. In immunofluorescence

assays, ten antibodies labelled the contents of the ventral vesicles, and two antibodies labelled the contents of the large peripheral vesicles in *P. nicotianae* zoospores. Five antibodies target the contents of a putative homologue to the dorsal vesicles in *P. nicotianae*. The ultrastructural localisation of some of the vesicle antigens was determined using immunogold labelling of ultrathin sections of *P. nicotianae* and *P. cinnamomi* zoospores. Immunoblotting revealed that the two antibodies directed towards the large peripheral vesicles recognised three polypeptides with a molecular weight larger than 200 kDa in crude protein extracts from *P. nicotianae* zoospores. Nine of the ten antibodies directed towards the ventral vesicles reacted on immunoblots; they recognised a proteinaceous epitope on an antigen with a molecular weight larger than 200 kDa in *P. nicotianae*. In *P. cinnamomi* they recognised a single, slightly smaller polypeptide with the same apparent molecular weight as that labelled by Vsv-1, a monoclonal antibody raised against *P. cinnamomi* spores. Immunocytochemical labelling showed that they cross-reacted with ventral vesicles in *P. cinnamomi* zoospores. The fact that these ventral vesicle antibodies reacted with a proteinaceous epitope makes them candidates for immunological screening of expression libraries.

In the fourth part of this project, the monoclonal antibodies raised in the previous part of the thesis and directed towards the ventral vesicles were used for the immunological screening of two cDNA expression libraries made from RNA of *P. cinnamomi* hyphae that had been induced to sporulate. A positive cDNA clone was purified, sequenced and the data analysed. The results showed that the sequence of the cDNA clone was similar to that of one group of secreted animal adhesives, namely the thrombospondin type I repeat proteins. The cDNA clone was used to purify a genomic clone of this gene. The full sequence of the *Vsv* gene, as well as partial sequence of surrounding genes, was obtained from subclones of the genomic clone. Translated database searches found homologous sequences in animal proteins of the thrombospondin type 1 repeat family. Analysis of the inferred amino acid sequence of the encoded protein showed that it consists of an N-terminal signal sequence, a region of low complexity, 47 repeats containing conserved amino acid residues similar to those present in thrombospondin type 1 repeat proteins

in animal cells, and a C-terminal extension that is unrelated to any other characterised eukaryotic sequence. Analysis of genomic DNA using Southern blotting revealed the presence of two copies of the gene in *P. cinnamomi*; the second copy was weakly recognised. In *P. infestans* and *P. nicotianae* only one copy of the gene was recognised by the *P. cinnamomi* cDNA insert that was used as a probe.

The last part of this study was aimed at verifying that the protein that is recognised by the monoclonal antibodies against the ventral vesicles corresponds to the predicted Vsv protein. In an immunological approach, polyclonal immunoglobulin G antibodies were purified on a membrane coated with the fusion protein expressed by the cDNA clone. When these monospecific antibodies were tested in indirect immunofluorescence assays on *P. cinnamomi* zoospores, the large peripheral but not the ventral vesicles were labelled. However, results from peptide mass fingerprinting and N-terminal sequencing indicate that the protein indeed is the one encoded by the *Vsv* gene.

The work presented in this thesis is important for our understanding of zoospore adhesion in *Phytophthora*. It is also the first report of the successful cloning of an adhesive in a propagule of a plant pathogenic fungus. The results of this study could form the basis for the development of control measures targeting the pathogen adhesive.

List of abbreviations

AFLP	Amplified fragment length polymorphism
ALS	Agglutinin-like sequence
approx.	Approximate, approximately
AS	Ammonium sulphate
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
ConA	Concanavalin A
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetraacetic acid
ESI	Electrospray ionisation
EST	Expressed sequence tag
FITC	Fluorescein isothiocyanate
GAM-Au10	Goat anti-mouse antibody conjugated to gold particles with a diameter of 10 nm
GFP	Green fluorescent protein
h	Hour(s)
IEF	Isoelectric focussing
IPTG	Isopropylthio-β-D-galactoside
IR	Infra red
IT	Ion trap
L	Litre
M	Molar
MALDI	Matrix-assisted laser desorption/ionisation
MES	2-[N-Morpholino]ethanesulphonic acid
min	Minute(s)
MS	Mass spectrometry
MSS	Mineral salts solution
MTM	Macroconidial tip mucilage
NCBI	National Center for Biological Information
OD_{600}	Optical density at 600 nm
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate buffer saline
PBSBG	PBS with BSA and gelatine
PBST	PBS with Tween20
PCR	Polymerase chain reaction
PGI	*Phytophthora* Genome Initiative
PIPES	Piperazine-N,N'-bis(2-ethane sulfonic acid)
PNGase	Peptide N-glycosidase
ppm	Parts per million
PVDF	Polyvinylidene fluoride
RFLP	Restriction fragment length polymorphism
RT	Room temperature
s	Second(s)
SAM	Sheep F(ab')2 anti-mouse antibody
SARa	Sheep F(ab')2 anti-rabbit antibody
SCA	Stigma/stylar cysteine-rich adhesin
SDS	Sodium dodecylsulphate
STM	Spore tip mucilage
TAE	Tris-acetate/EDTA
TBS	Tris buffered saline
TBST	TBS with Tween20
TOF	Time-of-flight
TS1	Thrombospondin type 1
TSR 1	Thrombospondin type 1 repeat
UV	Ultra violet

Table of contents

Chapter 1 Structural characteristics of zoospores and molecular aspects underlying *Phytophthora* pathogenicity

The plant pathogen that causes late blight on potato was introduced into Europe in the 1840s when it brought about the devastation of a large part of the potato crop all over Europe. Potatoes had become the staple food since their introduction into Europe by Spanish conquistadores in the 16th century and the destruction of the crop had disastrous consequences. It is estimated that in Ireland alone approx. 1 million people died of starvation and another million people chose to emigrate to the New World as a direct result of the famine that followed (Bourke 1964). In known history, no other pathogen has had such an impact on human life. At the time, Louis Pasteur's germ theory was in its infancy and the cause of the late blight disease was unknown. It was not until 1861, that Anton DeBary finally established that a fungus caused the late blight on potato and coined its name *Phytophthora* (literally: plant destroyer). DeBary's work in proving that *P. infestans* was responsible for the late blight disease on potato plants was a watershed as not only did it reveal the cause of the potato disease, it also pioneered the whole field of plant pathology (Berkeley 1846 and DeBary 1876 as cited in Ristaino 2002).

Costs for control measures and crop losses due to *P. infestans* diseases are an estimated 5 billion US dollars per year. But *P. infestans* is not the only *Phytophthora* species that causes significant losses. Other *Phytophthora* species, such as *P. cinnamomi* and *P. nicotianae*, have also large impacts world wide as they are each able to colonise over 1000 different plant species; horticultural, crop, and ornamental plants alike can be infected (Zentmyer 1980; Erwin and Ribeiro 1996). *P. cinnamomi* has shaped forests in Australia, Europe, and the USA by severely decimating susceptible species. In Western Australia, this pathogen was introduced in the 1920s but it took plant pathologists until the late 1960s to identify the pathogen (Newhook and Podger 1972). To date, neither breeding of resistant plants nor chemical controls have succeeded in fully protecting plants from *Phytophthora* diseases, possibly

because of the genetic flexibility of the Oomycetes that is likely to facilitate rapid adaptation to the imposed measures (Tyler 2001).

1.1 The phylogeny of *Phytophthora*

Today, about 60 species of *Phytophthora* are known, all of them being plant pathogens (Erwin and Ribeiro 1996). *Phytophthora* species are grouped within the Class Oomycetes which also includes *Achlya*, *Saprolegnia*, *Lagenidium*, and *Pythium* species (e.g. Beakes 1989, Patterson 1989 or Tyler 2001). Species of *Saprolegnia* are fish pathogens and species of *Lagenidium* are insect pathogens, and they also lead to large economic losses. For a long time, the Oomycetes were included in the taxonomic group of fungi, mainly because of their heterotrophic nature, growth by polarised hyphal extension, and reproduction by means of spores, all features these organisms share with true fungi. However, early mycologists recognised morphological and biochemical differences between the true fungi and the Oomycetes. For example, the mitochondria of Oomycetes have tubular cisternae and their cell walls contain little chitin – the main component of true fungal cell walls – but high amounts of β-glucans and cellulose. Recent genetic analyses have confirmed the separate position of these important pathogens. The Oomycetes are now included in the kingdom Stramenopila, a group of organisms that share the presence of flagellar tripartite tubular hairs. Other organisms included in this kingdom are the heterokont golden brown algae and diatoms (Sogin and Silberman 1998, also reviewed in Tyler 2001). The separate position of oomycete pathogens has to be considered when searching for control measures. An example for this is the often-cited azole fungicides that target ergosterol biosynthesis; they fail against Oomycetes since, in contrast to true fungi, they are devoid of this pathway and must obtain sterols from their host (e.g. Tyler 2001).

1.2 The life cycle of *Phytophthora*

The life cycle of *Phytophthora* species is similar to that of other Oomycetes (reviewed in Erwin and Ribeiro 1996). *Phytophthora* can reproduce sexually and asexually as shown in Fig. 1.1 for *P. cinnamomi*.

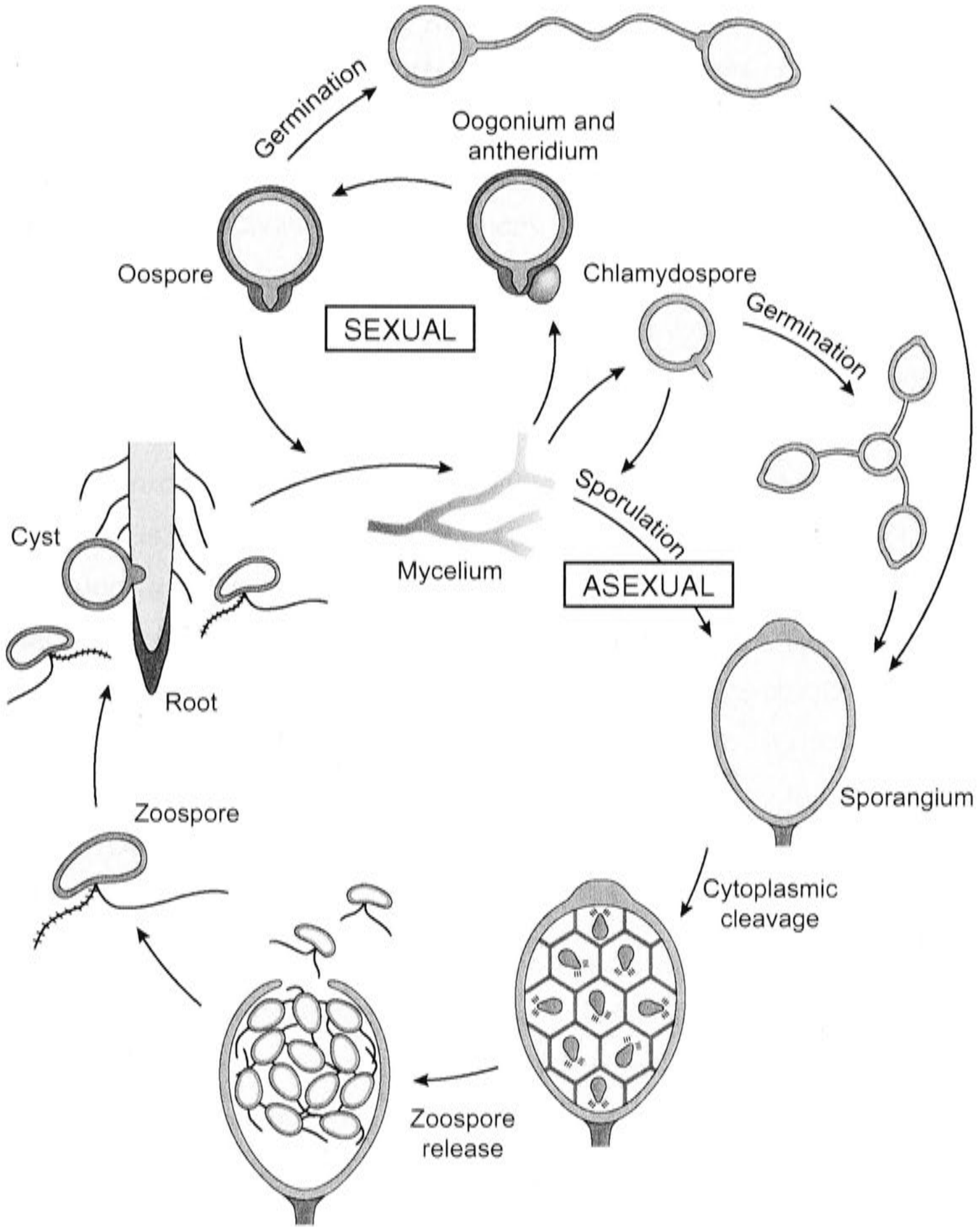

Fig. 1.1 Sexual and asexual life cycle of *P. cinnamomi*. During sexual reproduction the hyphal tip that will eventually form the female component (oogonium) grows through the one for the male component (antheridium). The developing antheridium subsequently forms a collar around the stalk of the oogonium. A hypha grows from the antheridium towards the oogonial cell wall, ruptures it, and releases the antheridial nucleus into the oogonial cytoplasm where karyogamy follows and a walled oospore is formed. Germination of the oospore can lead to new infections on susceptible host plants. During asexual reproduction multinucleate zoosporangia can be formed. Upon certain environmental triggers cytoplasmic cleavage of the sporangia leads to the production and release of uninucleate motile spores. These zoospores can encyst and germinate to infect a host plant. Under unfavourable conditions the hyphae also can produce thick-walled chlamydospores. Both oospores and chlamydospores can germinate and produce zoosporangia.

1.2.1. Sexual reproduction

When hyphae of opposite mating types in heterothallic species like *P. infestans*, *P. cinnamomi* or *P. nicotianae* meet, sexual reproduction can take place. In homothallic species like *P. cactorum* or *P. insolita* a second compatibility group is not needed for sexual reproduction. Antheridia (sing. antheridium, male component) and oogonia (sing. oogonium, female component) are produced. The position of the antheridia can be paragynous (next to the oogonium) or amphigynous. When amphigynous antheridia form, the tip of the differentiating oogonium grows through the developing antheridium. The antheridium subsequently forms a collar around the stalk of the multinucleate oogonium. The reduction division from diploid to haploid occurs within the antheridia and oogonia; this is different to the true fungi that are haploid most of their life cycle. All but one nucleus in the oogonium migrate to the cell periphery and disintegrate. A fertilisation tube grows from the antheridium towards the oogonium, ruptures the cell wall, and the antheridial nucleus migrates into the oogonium. In contrast to true fungi, Oomycetes never produce uninucleate gametes. Karyogamy follows the plasmogamy and an oospore develops. Oospores can overwinter in infected plant tissue or soil and give rise to infections in the following year (Drenth et al. 1995).

The occurrence of the second mating type seems to be an important issue in the case of *P. infestans*. It has been claimed that before the 1980s, only the A1 mating type was found outside Mexico. After that time, the A2 mating type could be detected in increasing numbers in Europe (Hohl and Iselin 1984, Drenth et al. 1993a) and in the USA (Deahl et al. 1991); since then the genetic diversity of *P. infestans* has increased (Drenth et al. 1993b, Zwankhuisen et al. 1998) as has the number of fungicide resistant strains (Gisi and Cohen 1996). However, recently, investigation of herbarium material revealed that oospores were already present in samples collected as early as 1876 (Ristaino 1998 as reviewed in Ristaino 2002).

1.2.2. Asexual reproduction

Of great importance for the development of *Phytophthora* diseases is the ability to reproduce asexually. Two types of asexual spores can be made: chlamydospores and zoosporangia. Chlamydospores are important as a source of inoculum because they can withstand harsh environmental conditions (reviewed in Erwin and Ribeiro 1996). However, the rapid spread of *Phytophthora* diseases and the devastating effects as seen in the Great Famine in the 19th century are most likely due to the production of sporangia. Sporangia can either germinate directly, or even more important for directed short-range dispersal, they can germinate indirectly and produce a number of zoospores which are released from the sporangia. Sporangial cleavage into individual zoospores can be induced by environmental conditions that favour disease development, the most important factor probably being the availability of free water. Zoospores are thought to represent the main infective agent and are the subject of the research carried out in this thesis. They can actively swim towards new plants to which they can be guided by chemotactic gradients (Cameron and Carlile 1978, Khew and Zentmyer 1973, Morris and Ward 1992) or electric fields (van West et al. 2002). Once they are in the vicinity of plant roots they encyst, a process that has been shown to be dependent on calcium fluxes across the zoospore membrane in *P. parasitica* (Warburton and Deacon 1998). During encystment, the zoospores retract or detach their flagella, release an adhesive that attaches them to the plant tissue, and form a thick cell wall. The process of encystment is completed within a few minutes. The cysts can germinate within 20-30 min after encystment, and the hyphae quickly colonise the host. Once the nutrients in the host tissue are depleted, the hyphae grow to the plant surface, produce new sporangia, and release more zoospores. The asexual life cycle can be completed within days and can be repeated multiple times within a single crop-growing season.

1.3 Structural characteristics of *Phytophthora* zoospores

Phytophthora zoospores are kidney-shaped, wall-less cells bearing two flagella. The flagella, which are responsible for zoospore motility, emerge from a groove on the slightly flattened side of the spore that is termed the ventral surface. Both flagella are formed by plasma membrane enclosed axonemes, microtubular structures that follow the (9+2) building plan of eukaryotic flagella and cilia. One of the flagella is directed anteriorly, the other one posteriorly. The longer posterior flagellum has only a few non-tubular appendages, and is therefore termed the smooth or whiplash flagellum (Hardham 1987a). It appears to function as a rudder during swimming of the zoospore. The anterior flagellum is shorter than the posterior flagellum and carries two opposite rows of tripartite tubular hairs characteristic of the Stramenopiles (Hardham 1987a) and called mastigonemes. The mastigonemes have been implicated in the thrust reversal of the anterior flagellum that pulls the cell through the medium (Jahn et al. 1964, Cahill et al. 1996). So far, no data on their molecular composition are available.

Serial sectioning of two zoospores of *P. cinnamomi* has revealed the high degree of structural organisation within the spore (Hardham 1987b). The nucleus is pear-shaped in cross-section and dominates the cytoplasm. Its narrow end points towards the ventral groove. Between the nucleus and the plasma membrane lining the groove are the two basal bodies from which the flagella emerge. Three flagellar rootlets composed of microtubules arise from the vicinity of the basal bodies (Hardham 1987a). Immunolabelling with antibodies directed towards the calcium-binding molecule, centrin, has revealed that centrin filaments colocalise with the anterior flagellar rootlet and form a connecting fibre between the two basal bodies (Harper et al. 1995). Genes encoding α- and β-tubulin have been cloned from *P. nicotianae* (Dr. D. Skalamera and Prof. A.R. Hardham, personal communication) and *P. cinnamomi* (Weerakoon et al. 1998), respectively. Genes encoding centrin and the important flagellar axonmal components, dynein and radial spoke protein, have also been recently cloned from these *Phytophthora* species (P. Golletz and R. Narayan, personal communication). In *P. cinnamomi*, the calcium

binding protein, calmodulin, has also been localized within a swelling at the base of the anterior flagellum (Gubler et al. 1990).

In the anterior end of the zoospore, next to the nucleus, lies the water expulsion vacuole, a dynamic complex that functions to maintain the osmolarity of the zoospores at a physiological level. Vacuolar H^+-ATPase proteins have been localised to the membranous reticulum of the water expulsion vacuole in *P. nicotianae* (Mitchell and Hardham 1999), and their activity has been suggested to power the accumulation of proline within the reticulum where it may act as an osmolyte to withdraw water from the zoospore cytoplasm (Ambikapathy et al. 2002).

Much of the zoospore cytoplasm is taken up by large vesicles containing β-laminarin, an important carbohydrate store in oomycetes (Bartnicki-Garcia and Wang 1983). Ultrastructural studies of zoospores of *P. cinnamomi*, *P. palmivora*, *P. parasitica*, and *Pythium aphanidermatum* have shown the presence of mitochondrial profiles in the cell periphery (Hardham 1987b, Bimpong and Hickman 1975, Reichle 1969, and Grove and Bracker 1978). In *P. cinnamomi*, the profiles result from three to four large and 19-23 small mitochondria. The cell cortex of *Phytophthora* zoospores also contains four different types of vesicles that are spatially organised. Disc-shaped peripheral cisternae lie directly underneath the plasma membrane over most of the cell surface except within the groove. Large peripheral vesicles and dorsal vesicles are mostly confined to the dorsal cell cortex. Ventral vesicles occur predominantly underneath the ridges of the ventral groove. In samples of *P. cinnamomi* that were chemically fixed and subsequently processed for electron microscopy, the diameter of the elliptic large peripheral vesicles with a granular or flocculent content was approx. 0.6 x 0.8 µm, and that of the small peripheral vesicles was approx. 0.3 µm (Hardham 1987b, Hardham et al. 1991a, Hardham et al. 1994). Ventral vesicles contain plate-like inclusions after fixation with OsFeCN, dorsal vesicles have contents with electron-lucent areas. In *P. nicotianae*, the appearance of the contents of the ventral and large peripheral vesicles is the same as in *P. cinnamomi*. However, the contents of the dorsal vesicles lack electron-lucent areas. The diameter of all three types of vesicles is smaller in *P. nicotianae* than in *P. cinnamomi*: large peripheral vesicles

measure only 0.3 µm in diameter, the dorsal and ventral vesicles only approximately 0.2 – 0.25 µm (Hardham et al. 1994).

Peripheral cisternae and large and small peripheral vesicles have been found in secondary zoospores of most Oomycetes investigated so far (e.g. Beakes 1987, Grove and Bracker 1978, Reichle 1969), and much research has focussed on them as their contents are thought to be directly involved in plant infection. Proposed functions for the contents of the different types of peripheral vesicles include cyst coat formation, adhesion, nutrition, and cyst wall formation (Gubler and Hardham 1990, Burr and Beakes 1994, Hardham 1995). In *P. cinnamomi*, monoclonal antibodies directed towards the peripheral cisternae are available (e.g. Cpw-1, Hardham et al. 1991b, Gubler and Hardham 1991). They label the lumen of the cisterna in zoospores and the cell wall in cysts. On immunoblots, Cpw-1 antibodies react with a smear of polypeptides (Hyde et al. 1991).

It was initially thought that the contents of the large peripheral vesicles were secreted and constituted the adhesive in *Phytophthora* and *Py. aphanidermatum* zoospores (Sing and Bartnicki-Garcia 1975a and 1975b, Estrada-Garcia et al. 1990a). However, fixation of *Phytophthora* zoospores with formaldehyde fixatives can cause the large peripheral vesicle membranes to rupture and subsequently, their contents are released into the surrounding medium during sample handling. The generation of monoclonal antibodies directed towards the contents of the large peripheral vesicles of *P. cinnamomi* (Hardham et al. 1986) and their use in immunocytochemical labelling revealed the fact that in well-preserved zoospores, the contents of the large peripheral vesicles were not secreted during encystment, but instead the large peripheral vesicles moved away from the plasma membrane and became randomly distributed within the cysts (Gubler and Hardham 1988). These Lpv antibodies labelled the contents of the large peripheral vesicles in indirect immunofluorescence assays on *P. cinnamomi*, *P. parasitica*, *P. nicotianae*, *P. hevea*, and *P. megasperma* zoospores that were fixed with formaldehyde (to render them permeable to the antibody; Fig.1.2A) showing conservation of the epitope(s) amongst various *Phytophthora* species. Labelling of large peripheral vesicles was also detectable in indirect immunogold assays (see Gubler and

Hardham 1988 and 1990). On immunoblots of crude protein extracts from lyophilised zoospores the Lpv-1 monoclonal antibody recognised three high molecular weight glycoproteins (larger than 500 kDa, see Fig. 1.3A, Gubler and Hardham 1990). Further studies showed that the large peripheral vesicles became dilated after cyst germination and their contents appeared to be degraded as germling growth proceeded (Gubler and Hardham 1990). This behaviour indicates a role in nutrient supply during early infection (Gubler and Hardham 1990).

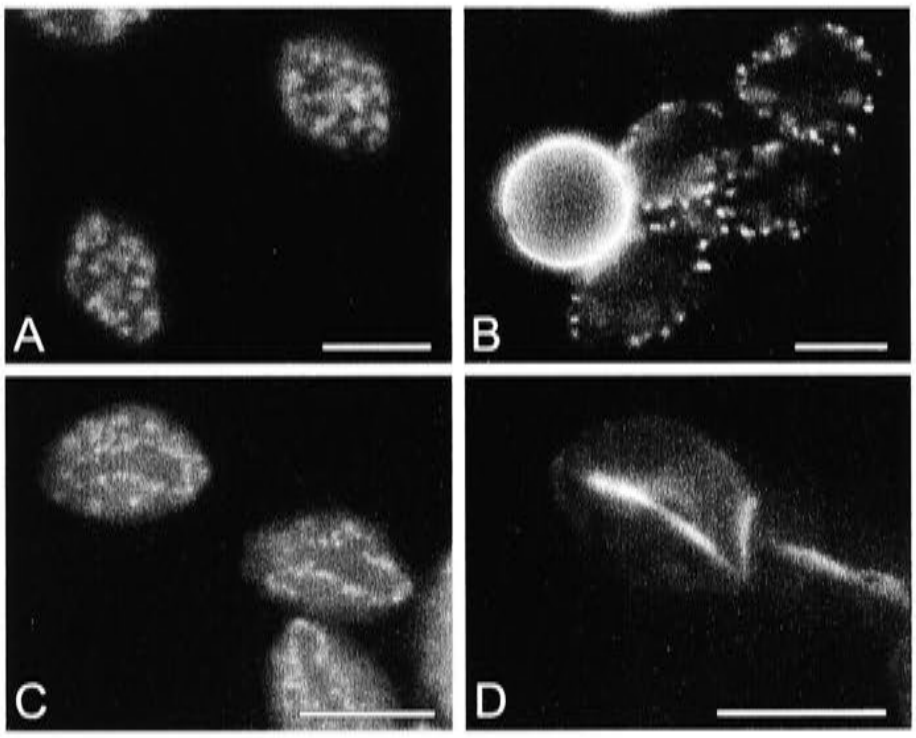

Fig. 1.2 Immunofluorescence micrographs of zoospores and cysts fixed with formaldehyde and labelled with monoclonal antibodies towards peripheral vesicles. In A, B, and D zoospores or cysts of *P. cinnamomi* were immunolabelled with monoclonal antibody Lpv-1, Cpa-2, and Vsv-1, respectively. In C zoospores of *P. nicotianae* were labelled with monoclonal antibody Vsv-1. In A the large peripheral vesicles are strongly fluorescent, in B the dorsal vesicles are fluorescent. In C the ventral vesicles are labelled and in D the young cysts show fluorescence on the surface that corresponds to the ventral surface of the zoospore after release of the putative adhesive. The micrograph in B is courtesy of Prof. A. R Hardham.

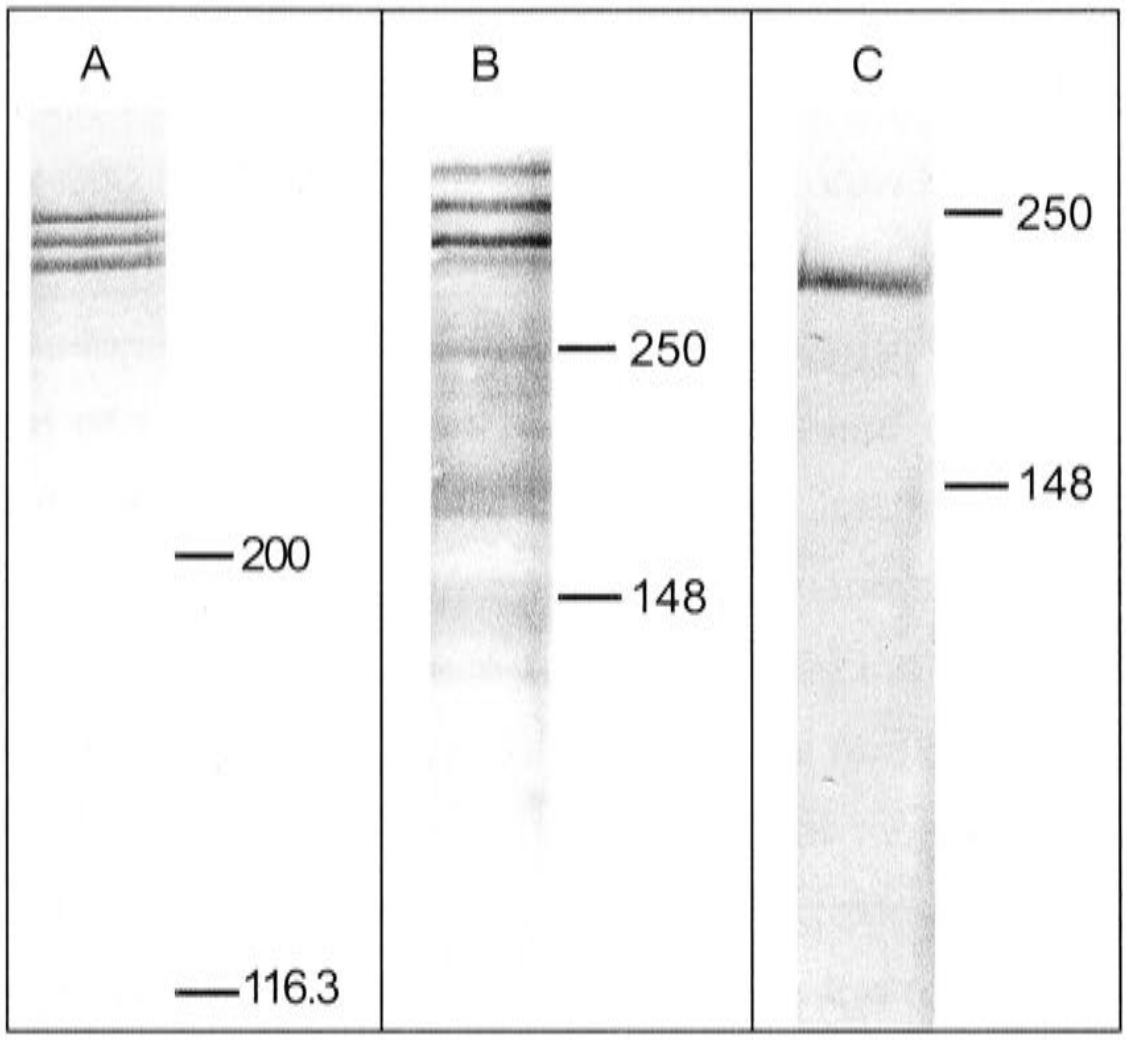

Fig. 1.3 Immunoblots of *P. cinnamomi* zoospore proteins labelled with monoclonal antibody Lpv-1 (A), Cpa-2 (B), and Vsv-1 (C). Three high molecular weight proteins are labelled with Lpv-1, another set of three high molecular weight proteins with Cpa-2, and a single polypeptide with a relative molecular weight of approx. 220 kDa with Vsv-1. Numbers on the right indicate position of molecular weight markers in kDa.

The two types of small vesicles, ventral and dorsal vesicles, are of a similar size and in early studies were not distinguished by conventional transmission electron microscopy (e.g. Hardham 1987b). Only the generation of monoclonal antibodies towards their contents made it possible to discern the two types (Fig.1.2B and C). Initially, only antibodies directed towards the contents of the dorsal vesicles, Cpa antibodies, were available (Hardham et al. 1986). Cpa-2 labelled three high molecular weight proteins in immunoblots (larger than 300 kDa) and had a glycoprotein epitope (Fig. 1.3B, Gubler and Hardham 1988, Hardham and Gubler 1990). Studies showed that the antigen was secreted during encystment of the zoospores and it was thought that the dorsal vesicles were involved in adhesion of the cysts. However, adhesion assays using the Cpa-2 antibody showed no reduction of adhesiveness upon addition of the antibody (Prof. A.R. Hardham, personal communication). Later, antibodies towards the contents of the ventral vesicles, Vsv antibodies, were generated

(Fig. 1.2C and D, Hardham and Gubler 1990, Hardham et al. 1991b). The Vsv antigen is also secreted upon encystment of the zoospores (Fig. 1.2D, Hardham and Gubler 1990). On immunoblots of *P. cinnamomi* zoospore extracts a single protein of over 200 kDa is recognised by antibody Vsv-1 (Fig. 1.3C). Antibody binding is only abolished after pronase treatment indicating the proteinaceous nature of the epitope of Vsv-1 (Hardham and Gubler 1990). It was realised that the localisation of the Vsv antigen between plant and cyst surface made the contents of the ventral vesicles prime candidates for the adhesive, especially when it was observed that *P. cinnamomi* zoospores predominantly orient themselves with the ventral surface facing towards the host root immediately before encystment as do zoospores of *Saprolegnia ferax* (Hardham and Gubler 1990, Lehnen and Powell 1989). Ultrastructural localisation of the Cpa-2 antigen showed it on the dorsal surface of encysting zoospores (i.e. facing away from the plant surface) and subsequently a role in the formation of a cyst coat was suggested (Hardham and Gubler 1990).

The antigens of the Lpv, Cpa, and Vsv antibodies are detectable within hyphae that have been depleted of nutrients and are preparing for sporulation *in-vitro* and *in-planta* (Dearnaley and Hardham 1994, Chambers et al. 1995). All the antigens can also be found in chlamydospores; these can germinate to form zoosporangia (Dearnaley et al. 1996). In oospores, only the antigens of antibody Lpv-1 can be detected (Chambers et al. 1995). The only antigen that has been characterised at all on a molecular level is the Lpv-1 antigen. The 3'-half (about 6 kbp) of the gene encoding this antigen has been cloned and sequenced (Marshall et al. 2001). As it is apparent that the contents of the peripheral vesicle play an important role in plant pathogenesis, one of the main aims of this thesis was to extend the characterisation of the peripheral vesicle contents at the molecular level.

1.4 Molecular biology of *Phytophthora*

Molecular analysis of Oomycetes is hampered by their diploid nature (e.g. Erwin and Ribeiro 1996). Additionally, transformation techniques have been available only since the 1990s (Judelson et al. 1991; Bailey et al. 1991). Since then,

improvements have been made in the transformation of *P. infestans*, and more *Phytophthora* species have been transformed (e.g. *P. megasperma* f. sp. *glycinea*, Judelson et al. 1993a), yet transformation is still not a routine procedure for *Phytophthora* species (Dr. J. Marshall and Dr. W. Shan, personal communication). However, genes have been silenced in *P. infestans* using antisense inhibition (Judelson et al. 1993b, van West et al. 1999a). In order to facilitate large scale analysis of the genomes of these destructive plant pathogens and to find out about the interactions with their host plants the *Phytophthora* Genome Initiative (PGI) was founded. Expressed sequence tag (EST) clones from a variety of developmental and infection stages were generated from *P. sojae* and *P. infestans* and sequenced (Kamoun et al. 1999, reviewed in Tyler 2001). The data obtained by the PGI were analysed, stored, and made accessible for *Phytophthora* researchers (Waugh et al. 2000, Qutob et al. 2000). Currently, 41000 more EST clones from *P. sojae* and 14000 from *P. infestans* are being sequenced (Tyler 2001). Genomic libraries have been generated for a number of species of *Phytophthora* including four bacterial artificial chromosome (BAC) libraries for *P. infestans* (Randall and Judelson 1999; Whisson et al. 2001), *P. nicotianae* (Shan and Hardham, manuscript submitted), and *P. sojae* (Arrendondo et al. 1997). The entire 62 Mbp genome of *P. sojae* has now been sequenced to create a publicly available reference map for other oomycete species. Within the Hardham laboratory, the differential screening of over 25000 cDNA clones from *P. nicotianae* and *P. cinnamomi* has led to the identification and sequencing of over 500 different genes whose expression is up-regulated in zoospores, in germinating cysts or during sporulation (Prof. A.R. Hardham, personal communication). Many of these genes (approx. one third) have to date no homologues in the non-redundant database (*P. nicotianae*, Dr. D. Skalamera, personal communication; *P. cinnamomi*, R. Narayan, personal communication). This makes it necessary to characterise the gene products on an ultrastructural and biochemical level. In 1999, green fluorescent protein (GFP) constructs were used to transform *P. palmivora* and *P. parasitica* var. *nicotianae*, making it easier to enter the exciting area of functional genomics (van West et al. 1999b, Bottin et al. 1999). In addition to the sequence information that is available, genetic maps for *P. infestans* and *P. sojae* have been generated. Van der Lee et al. (1997)

published a genetic map of *P. infestans* employing a total of 183 amplified fragment length polymorphism (AFLP) markers and seven restriction fragment length polymorphism (RFLP) markers. The mating type locus could also be located on the genetic map. Using bulked segregant analysis, the avirulence genes Avr1, Avr3, Avr4, Avr10, and Avr11 could be placed on the map (van der Lee et al. 2001). A genetic map of *P. sojae* was generated and subsequently updated using a total of 35 RFLP, 105 AFLP, and 236 randomly amplified polymorphic DNA markers; 10 avirulence genes could be placed on the map (Whisson et al. 1995, May et al. 2002). It is hoped that the wealth of information that has been generated in the last decade will soon shed light on the interaction of *Phytophthora* species with their host plants, hopefully making it possible to find a way to overcome the threat that these pathogens pose on plants.

1.5 Objectives and experimental approaches

The objective of the study of this thesis was to obtain information on the molecular bases of *Phytophthora* pathogenicity. The emphasis was on zoospore components important for the spread of disease. The two species used in this thesis, *P. cinnamomi* and *P. nicotianae*, were chosen for the following reasons: firstly, all stages of the life cycle could be obtained *in-vitro* using well-established protocols. Secondly, a wide range of monoclonal antibodies towards zoospore components implicated in plant pathogenicity in the two species was available. More antibodies could be generated building on the knowledge gained in previous studies. Thirdly, cDNA expression libraries for both species were available. In the case of *P. cinnamomi*, two cDNA expression libraries from hyphae that had been induced to sporulate had been made before I started my project and in the case of *P. nicotianae* a zoospore cDNA library was being generated by Dr. D. Skalamera, a member in our laboratory. The latter was soon available for immunological screening. Finally, genomic libraries for both species were already present or being made. Many of the studies reported in this thesis were aided by the fact that the isolate of *P. nicotianae* used in this project routinely produced about 10^6 zoospores mL^{-1}, a

value about ten times greater than achieved with *P. cinnamomi*. Both *P. nicotianae* and *P. cinnamomi* have a wide host range. Based on their ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA tandem they are currently placed into clades 1 and 7 within the *Phytophthora* cluster, respectively (Cooke et al. 2000).

The experimental approaches taken were as follows. In the experiments in Chapter 2, immunological screening of a *P. nicotianae* zoospore cDNA expression library was carried out to find a gene encoding a structural protein involved in the tripartite tubular hairs on the anterior flagellum. DNA sequencing of the positive clone followed its purification and the molecular data were analysed. The experiments in Chapters 3 and 4 were carried out to find monoclonal antibodies that could be used in cloning and sequencing of components of the vesicular compartment of *Phytophthora* species. In the experiments in Chapter 3, monoclonal antibodies directed towards zoospore components of *Lagenidium giganteum* were screened on *P. cinnamomi* zoospores hoping to find antibodies that cross-react with *P. cinnamomi*, but also to shed light on ultrastructural aspects of zoospores of *L. giganteum*. The experiments in Chapter 4 were aimed at generating and characterising monoclonal antibodies directed towards *P. nicotianae* zoospore components. These antibodies were also tested on *P. cinnamomi* to see whether all resources in the laboratory could be employed in the subsequent molecular approaches. The experiments in Chapter 5 include the immunological screening of two *P. cinnamomi* cDNA expression libraries and the cloning, sequencing, and characterising of the *Vsv* gene. The experiments in Chapter 6 were directed at verifying that the protein recognised by the monoclonal antibodies used for the cloning of the *Vsv* gene is indeed encoded by the cloned gene. For this, peptide mass fingerprinting and N-terminal sequencing were carried out; an immunological approach was also taken.

Chapter 2 Characterisation of a *Phytophthora nicotianae* flagellar surface antigen

2.1 Introduction

As described in Chapter 1, the two main zoospore functions that were the focus of my studies were zoospore motility and zoospore adhesion. This chapter describes that part of my thesis research that was directed towards an analysis of a flagellar surface component that plays an important role in zoospore motility. Flagella (or cilia as they are often called when occurring in large numbers) are present on cells of most major eukaryotic taxonomic groups, often only during certain developmental stages. The components of flagella are highly homologous amongst the various organisms in which they occur – even at a molecular level. Cilia or flagella are involved in the generation of a liquid current over the surface of cells, as in the mammalian respiratory tract; they are involved in cell-cell recognition events such as during mating in the green alga *Chlamydomonas*, and they function in perception of environmental signals, e.g. in *Chlamydomonas* or *Caenorhabditis elegans* (e.g. El Zein et al. 2003, Pan and Snell 2000, Bloodgood and Salomonsky 1990, Cole et al. 1998). The most obvious function of flagella and cilia is the propulsion of single cells, as is the case in *Phytophthora* zoospores.

2.1.1. The structure and functions of flagella

Flagella are specialised structures that are composed of an axoneme, the flagellar membrane, and the flagellar matrix. The axoneme consists of nine outer doublet microtubules and (usually) two central microtubules giving the flagellum rigidity and flexibility. The outer doublet microtubules are formed by one complete microtubule (the A-tubule) and one partial microtubule (the B-tubule). A multitude of other protein components is also involved in building the

axoneme. Specific functions of axoneme proteins are currently being unravelled.

Chlamydomonas reinhardtii is one of the most thoroughly investigated organisms regarding flagellar structure and function (for a recent review see Silflow and Lefebvre 2001). As in other organisms, a basal body is found at the base of each flagellum. A second partial microtubule (the C-microtubule) is added to the outer doublet microtubules forming the walls of the basal body. The flagellar matrix has lately received much attention. In 1993, Kozminski et al. observed intraflagellar transport in *C. reinhardtii* in both anterograde and retrograde directions using differential interference contrast microscopy. Since then, intraflagellar transport has been observed in flagella of other organisms including *C. elegans* (for a review see Rosenbaum and Witman 2002). It has been demonstrated that intraflagellar transport is responsible for the proper assembly and function of flagella.

The flagellar membrane is continuous with the membrane over the rest of the cell body, yet some membrane components are only present in this part of the membrane. It has been shown in *C. reinhardtii* that agglutinins necessary for mating of gametes are exclusively present on the flagellar membrane (Adair et al. 1983). In trypanosomatid protozoa the flagellar membrane has been recognised as a membrane domain that differs from the rest of the cell membrane: certain isoforms of glucose transporters, adenylate cyclases, and LDL receptors localise specifically to this membrane domain (for reviews see Bloodgood 2000, Landfear and Ignatushchenko 2001). A monoclonal antibody, Zf-1, raised against *P. cinnamomi* zoospores has also demonstrated that the Zf-1 antigen is confined to the flagellar membrane domain (Hardham et al. 1986).

2.1.2. Flagellar appendages

Various appendages can be present on flagella: flagellar scales as, for example, in *Pyramimonas gelidocola* (Chlorophyta, e.g. McFadden and Wetherbee 1985) or flagellar hairs. The latter include non-tubular hairs, hair scales, and bipartite or tripartite tubular hairs. Tripartite tubular hairs

(mastigonemes) consisting of a tapered base, a tubular shaft, and a number of terminal filaments are present in two opposite rows on the anterior flagellum of the asexual zoospores of oomycete species (reviewed in Dick 2001). In the case of *P. cinnamomi*, two terminal filaments are present on the tripartite hairs that are 1.6 µm long and have a diameter of 17 nm (Hardham 1987a). It is believed that the mastigonemes are inserted through the flagellar membrane and are attached to the flagellar axoneme. It has been suggested that rigid hairs like the tripartite tubular mastigonemes that are present on the flagella of single cells are important for the direction of their movement (Jahn et al. 1964). Using high-speed cinematography of particle movement the authors investigated the swimming behaviour of two species of *Ochromonas*. They concluded that the anteriorly directed flagellum pulls the cell through the water, even though sinusoid waves move from the cell body towards the distal tip of the flagellum. The direction of movement is therefore in the same direction as the flagellar wave and the tubular hairs present on the flagellum in two opposite rows were suggested to cause the thrust reversal of flagellar beat. A mathematical evaluation of the problem was given by Holwill and Sleigh in 1967. However, direct experimental evidence for the importance of rigid hairs in the motility of single cells was lacking until 1996. Cahill et al. (1996) showed that a monoclonal antibody directed towards the tripartite tubular hairs of *P. cinnamomi*, Zg-4, could lead to clumping and eventually the loss of tripartite tubular hairs in live cells. The swimming speed of the zoospores decreased with increasing antibody concentration. In many cases a reversal of the swimming direction occurred, the beating pattern of the anterior flagella was in many cases altered to a figure of eight pattern, and the beating frequency increased strongly. Heath et al. (1970) have investigated the ultrastructure of *S. ferax* in detail and found that the tubular hairs on the zoospores are synthesised in the endoplasmic reticulum.

A number of monoclonal antibodies are available that recognise epitopes on the tripartite tubular hairs of *P. cinnamomi* or *P. nicotianae* (Hardham et al. 1986, Gautam et al. 1999, Robold and Hardham 1998). However, only monoclonal antibody Pn14B7 recognises its antigen on immunoblots; it reacts with a

(glycol-) protein with an approx. molecular weight of 40 kDa (Robold and Hardham 1998). Although the ultrastructure of the tripartite tubular hairs of Oomycetes has been studied (reviewed in Dick 2001), the proteins composing the hairs have so far not been characterised at a molecular level. Monoclonal antibody Pn14B7 had the potential to provide a first insight into the biochemical composition of these important structures. The work described in this chapter of my thesis was aimed at characterising the Pn14B7 antigen at a molecular level.

2.2 Materials and Methods

2.2.1. *Phytophthora* strain and culture conditions

P. nicotianae strain H1111 (ATCC accession no. MYA 141) was used for DNA and protein isolations. Zoospores of *P. nicotianae* were produced following the methods in Robold and Hardham (1998) with some modifications. Agar plugs containing hyphae were incubated on discs of miracloth (Calbiochem-Behring Corp., La Jolla, CA, USA) on V8 agar containing 10% cleared V8 juice (Campbell's Soups Aust. Pty Ltd., Lemnos, Aust.), 0.002% β-sitosterol, 0.01% $CaCO_3$, and 1.7% Bacto agar (Difco, Detroit, MI, USA) for 5-6 days at 25°C in the dark. The miracloth discs were transferred individually into Petri dishes containing approx. 10 mL V8 broth (5% cleared V8 juice, 0.002% β-sitosterol, and 0.01% $CaCO_3$) and grown in still cultures for 7-30 days at 23°C in the light. Cleavage of sporangia was induced by rinsing the miracloth discs with the adhering mycelia four to five times in cold distilled water. After the last rinse, between 10 and 20 mL of cold distilled water were added and zoospores were released during a 60-75 min incubation at 18°C on a light box.

2.2.2. Immunological screening of a *Phytophthora nicotianae* zoospore cDNA expression library

A cDNA expression library using mRNA isolated from *P. nicotianae* zoospores was constructed by Dr. D. Skalamera in λZAPII according to the supplier's manual (Stratagene, La Jolla, CA, USA). The screening of the library and part of the sequencing of the positive cDNA clone was carried out by an undergraduate student, Corinna Paeper, working under my supervision.

First round of screening

Approx. 3 x 10^5 phage particles were mixed with 3.6 mL *Escherichia coli* XL1 blue MRF' cells (OD_{600}=0.5), incubated for 15 min at 37°C, mixed with 6.5 mL NZY top agar (1% [w/v] NZ amine, 0.5% [w/v] NaCl, 0.5% [w/v] bacto-yeast extract, pH 7.5, 0.8% [w/v] agar) per plate, plated onto six Petri dishes with a

diameter of 150 mm (Sarstedt, USA) containing NZY agar (1.5% [w/v] agar), and incubated at 42°C for 4 h before overlaying the plates with nitrocellulose membrane circles (Hybond C, Amersham Pharmacia Biotech) wetted with 10 mM isopropylthio-β-D-galactoside (IPTG). The plates were incubated overnight at 37°C and cooled for at least 1 h at 4°C. The position of the membranes was marked, the membranes removed and rinsed in a solution containing 100 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween20 (TNT buffer) for 30 min. The agar plates were stored at 6-8°C until the result of the screen was known. After incubation in TNT buffer the membrane discs were treated in the same way as all other immunoblots.

Immunoblot

Non-specific binding to the membranes was blocked by incubation in Tris buffered saline (TBS; 150 mM NaCl in 10 mM Tris-HCl pH 7.4) containing 0.05% Tween20 (TBST) for 2 h at room temperature (RT). The membranes were rinsed three times for 5 min in TBST, incubated in primary antibody solution for 45-90 min on a horizontal shaker, rinsed again as before, incubated in secondary antibody solution as described for the primary antibody, rinsed twice in TBST, twice in TBS, and once in a 100 mM Tris-HCl solution pH 9.5 containing 100 mM NaCl and 50 mM $MgSO_4$. The primary antibody Pn14B7 (Robold and Hardham 1998) was used at a concentration of 20 µg mL^{-1} diluted in TBST. Sheep F(ab') anti-mouse antibody conjugated to alkaline phosphatase (SAM-AP; Silenus, Melbourne, Australia) diluted 1:10000 in TBST was used as a secondary antibody. The blot was developed by adding the alkaline phosphatase substrate (one Sigma Fast 5-bromo-4-chloro-3-indoyl phosphate/nitroblue tetrazolium tablet per 10 mL distilled water). The colour reaction was stoped by removing the developing solution and rinsing the membranes in two changes of distilled water. The membranes were dried between filter paper.

Plaque purification

When a positive signal was present in the first round of screening, an agar plug containing the positive phage (and possibly contaminating phage) was removed with the large end of a pasteur pipette, and the phage eluted overnight into

500 µL of a 50 mM Tris-HCl solution pH 7.5 containing 5.8 g NaCl, 2 g $MgSO_4x7H_20$, and 5 mL of a 2% gelatine stock solution per L (SM buffer) and 20 µL chloroform at 6-8°C. The phage stock was re-screened on Petri-dishes with a diameter of 90 mm until pure plaques were obtained.

Phagemid excision

After purification, the phagemid was excised and sequenced. *E. coli* XL1-blue MRF' and SOLR cells were grown overnight in LB broth (1% NaCl, 1% tryptone, and 0.5% yeast extract, pH 7.5) supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ at 30°C. The cells were collected by centrifugation at 1000 x g and resuspended in 10 mM $MgSO_4$ (OD_{600}=1.0). In a 14 mL propylene test tube, 200 µL *E. coli* XL1-blue MRF' cells, 250 µL phage stock and 1 µL ExAssist helper phage were incubated for 15 min at 37°C; 3 mL LB broth were then added and incubated for 3 h at 37°C with shaking. The suspension was heated to 70°C for 20 min and the cell debris removed from the excised pBluescript phagemid packaged as filamentous phage particles by centrifugation for 15 min at 1000 x g. The excised phagemid was grown on *E. coli* SOLR cells (10 or 100 µL phagemid solution plus 200 µL SOLR cells per dilution) on LB agar containing 50 µg mL^{-1} ampicillin after incubation of host cells and phagemid for 15 min at 37°C. The colonies that appeared on the agar contained the pBluescript double stranded phagemid. Single colonies of *E. coli* SOLR cells containing the phagemid were grown in 5 mL LB broth containing 75 µg ampicillin mL^{-1} for at least 5 h on a shaker. DNA was extracted using a DNA extraction kit (QIAprep® Miniprep 27104, QIAGEN).

2.2.3. Design of custom primers

Custom primers were designed using the program "Prime" on the server of the Australian National Genomic Information Service (WebANGIS; http://www.angis.org.au). The primers were between 18 and 22 nucleotides long and their position on the clone is shown in Figure 2.2. For a list of primers used see Appendix I. The primers were custom made by Sigma-Aldrich (Sydney, Australia).

2.2.4. DNA sequencing and analysis

DNA was sequenced with dye-terminators (Big Dye Reaction Mix, Perkin Elmer) and an Applied Biosystems automated fluorescent DNA sequencer. For the first sequencing reaction the RI Prox primer was used; for all other sequencing reactions custom primers were used. The sequencing reaction was carried out in a thermal cycler (one cycle at 96°C for 30 s, 25 cycles at 96°C for 15 s, 50 or 55°C for 5 s, and 60°C for 2 min, and one cycle at 30°C for 1 min). DNA was precipitated with 70% isopropanol.

The nucleotide sequence was translated into amino acid sequence using the program PepData on the WebANGIS server, and pattern searches done using the software SMART on the EMBL Heidelberg website (Schultz et al. 1998, Letunic et al. 2002; http://smart.embl-heidelberg.de/smart). Molecular weight and isoelectric point predictions were made using the program Compute pI/Mw on the ExPasy server (Bjellqvist et al. 1993; Bjellqvist et al. 1994; Wilkins et al. 1998; http://kr.expasy.org/tools/pi_tool.html). DNA, protein, and conserved domain database searches were carried out by the BLAST programs through the National Center for Biological Information (NCBI; Altschul et al. 1997; http://www.ncbi.nlm.gov/BLAST).

2.2.5. Production of polyclonal serum against components present in zoospores of *Phytophthora nicotianae*

Zoospores were prepared as detailed in section 2.2.1, fixed for 30 min at RT in 1% glutaraldehyde in 50 mM piperazine-N,N'-bis(2-ethane sulfonic acid) (PIPES) buffer pH 7.0, collected by centrifugation at 1000 x g for 3 min, rinsed once with 100 mM PIPES buffer pH 7.0 and twice with phosphate buffered saline (PBS; 20 mM sodium phosphate buffer containing 150 mM NaCl), and finally resuspended in 1 mL PBS. Between 3 and 5 x 10^6 zoospores mL^{-1} were obtained (sample a) and 500 µL of the suspension were ground in liquid nitrogen (sample b). The two samples were injected into two different rabbits and booster injections were given twice, after two and five weeks. Finally serum was tested on aldehyde fixed zoospores in indirect immunofluorescence assays as detailed in section 2.2.6, serum obtained, and stored at -70°C.

2.2.6. Immunofluorescent labelling of *Phytophthora nicotianae* zoospores

Indirect immunofluorescence labelling was carried out as described in Hardham et al. (1991b) with some modifications. *P. nicotianae* zoospores were fixed in 4% paraformaldehyde or in 4% formaldehyde combined with 0.2% glutaraldehyde in 50 mM PIPES buffer pH 7.0 for 30 min at RT. The cells were collected by centrifugation at 1000 x g for 2 min and the cell pellets washed once in 100 mM PIPES pH 7.0 and twice in PBS. The final samples were resuspended in distilled water, 15 µL aliquots were applied to each well of Multitest slides (ICN Biomedicals, Inc., USA), and allowed to air dry for approx. 1 h at 37°C. The cells were rehydrated in PBS and incubated in 15 µL primary antibody. The polyclonal rabbit serum was diluted 1:500 in PBS containing 1% bovine serum albumin (BSA) and 0.1% gelatine (PBSBG). After incubation, the wells were rinsed twice with PBS and incubated in 15 µL droplets of sheep F(ab')2 anti-rabbit immunoglobulin antibody conjugated to fluorescein isothiocyanate (SARa-FITC; Silenus, Melbourne, Aust.) diluted 1:20 or 1:30 in PBS. Both antibody incubations were for 45–70 min at 37°C. After two rinses in PBS and one in distilled water the cells were mounted in a glycerol based mounting medium (10% Mowiol 4-88 [Hoechst, Aust. Ltd., Melbourne] and 30% glycerol in 200 mM Tris-HCl pH8.5) containing 0.1% paraphenylene diamine and left to set at 4°C in the dark.

The cells were examined for immunofluorescence on a Zeiss Axioplan microscope equipped with FITC filters.

2.2.7. Immunopurification of the Pn14B7 antigen and N-terminal sequencing

A protocol for the immunopurification of the Pn14B7 antigen had to be developed.

Initially, it was attempted to purify the antigen with magnetic or agarose beads conjugated to protein G. Finally, a modification of the method by Olmsted

(1981) using membrane-adsorbed antibody was employed. A flow chart of the procedure is shown in Figure 2.1.

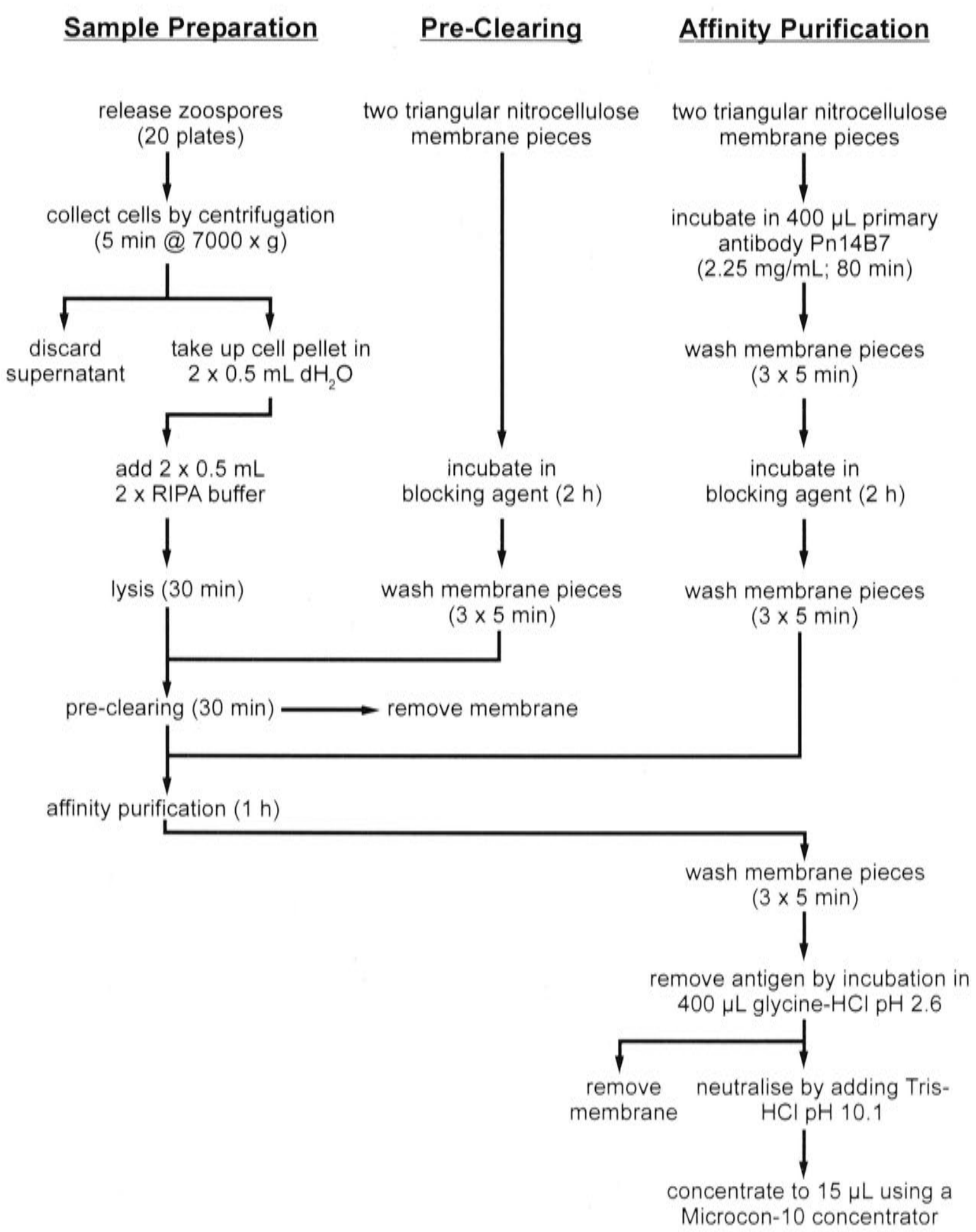

Fig. 2.1 Flow-chart of the procedure employed to immunopurify Pn14B7 antigen from a crude protein sample of *P. nicotianae* zoospores. For further details, see text.

Preparation of pre-clearing and immunopurification membranes

Two pre-clearing membranes and two immunopurification membranes were prepared. A pre-clearing step was included to minimise non-specific binding to the immunopurification membranes. Four triangular pieces of nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech) 1.0 cm wide at the base and 3.0 cm long were cut. The immunopurification membranes were incubated for 80 min in 400 µL of primary antibody solution (2.25 mg Pn14B7 mL^{-1}) in TBST and rinsed three times for 5 min in TBST. All four membrane pieces were then incubated for 2 h in TBST containing 5% skim milk powder and rinsed three times for 5 min in TBST.

Sample preparation and immunopurification

The sample was prepared by releasing zoospores from 20 plates as detailed in section 2.2.1 and collecting the cells for 5 min at 7000 x g after which time most of the cells were still zoospores. The pellets were resuspended at 6-8°C in a final volume of 1.0 mL distilled water and divided into two 500 µL aliquots. Immediately, 500 µL cold double strength RIPA lysis buffer were added to give a final concentration of single strength RIPA lysis buffer (150 mM NaCl, 1% NP 40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8.0, Harlow and Lane 1999). The cells were lysed for 30 min on a rotary shaker at 6-8°C, pre-cleared with the pre-clearing membranes for 30 min, and the immunopurification carried out for 60 min. The immunopurification membranes were removed from the sample, washed three times for 5 min, and combined into a new test tube. The antigen and some of the antibody present on the membrane pieces were eluted into 400 µL of glycine-HCl pH 2.6 for 10 min by placing the vial against a vortex mixer. The membrane pieces were removed from the tube and the solution neutralised by adding the appropriate amount of Tris-HCl pH 10.1. The sample was concentrated to a final volume of 15 µL using a Microcon 10 concentrator (Millipore), separated by electrophoresis on a 10% sodium dodecylsulphate-polyacrylamide gel (SDS-PAGE, Laemmli 1970), and blotted for 5 h onto a polyvinylidene fluoride (PVDF) membrane (Immobilon P®, Millipore). The proteins on the membrane were stained with 0.1% Coomassie Brilliant Blue dissolved in 45% methanol and 10% acetic acid for 1 min and destained in 45% methanol and 10% acetic acid for 10 min. The appropriate

band was cut out of the membrane, sent to the Biomolecular Recource Facility at the John Curtin School of Medical Research at the Australian National University, and N-terminal sequencing was carried out on an Applied Biosystems Procise Sequencer using standard procedures.

2.2.8. Two dimensional-gel electrophoresis

Freeze-dried *P. nicotianae* zoospores were solubilised in 2D-gel electrophoresis sample buffer pH 3-10 (5 M urea, 2 M thiourea, 4% CHAPS, 0.2% Pharmolytes pH 3-10, 40 mM Tris-Cl pH 6.8, and 65 mM dithiothreitol), the protein concentration estimated, and an isoelectric focussing (IEF) strip (Amersham Pharmacia Biotech) rehydrated overnight in 125 µL of the sample diluted in 2D-gel electrophoresis sample buffer pH 3-10 to give a protein amount of 50 µg. The proteins were separated for 30 min at 150 V and for 30 min at 300 V before ramping up the voltage to 3500 over a period of 5 h and focussing the proteins for 39 h at 3500 V. The IEF strip was treated for 15 min in a solution containing 0.2% DTT and 15 min in a solution containing 0.25% iodoacetamide before separation of the proteins on a 10% SDS-PAGE as a second dimension, and staining with Coomassie Brilliant Blue (section 2.2.9) or silver nitrate (section 2.2.10).

2.2.9. Coomassie Brilliant Blue staining of proteins after sodium-dodecyl sulphate-polyacrylamide gel electrophoresis

After gel electrophoresis one of the polyacrylamide gels was stained with Coomassie Brilliant Blue: the proteins were fixed in a solution containing 40% methanol and 10% acetic acid for 30 min, after this incubated for 15 min in the fixative containing 0.1% Coomassie Brilliant Blue dye (R-250; BioRad). To destain the gel it was incubated in several changes of fixative until protein spots were clearly visible.

2.2.10. Silver staining of proteins after sodium-dodecyl sulphate-polyacrylamide gel electrophoresis

One of the polyacrylamide gels was silver stained after 2D-gel electrophoresis. The method was based on a protocol by Blum et al. (1987). The gels were incubated overnight in 10% acetic acid and 30% ethanol after changing the solution once after 30 min. The gels were rinsed in 20% ethanol for 20 min and in distilled water for 10 min. They were soaked in a solution containing 0.02% sodium thiosulphate (sensitiser solution) for 1 min and rinsed three times for 20 s in distilled water. The gels were left on the shaker in a solution containing 0.1% silver nitrate for 45 min, rinsed three times for 5-10 s in distilled water, and developed in a solution containing 30 g sodium carbonate, 0.005% sodium thiosulphate, and 250 µL of a 37% formaldehyde solution per L. The developing reaction was stopped by adding 5% acetic acid. The gel was left in the stopping solution for 30 min and then dried onto filter paper.

2.2.11. Processing of images

Immunoblots and micrographs were scanned and edited using the Adobe Photoshop 6.0 software.

2.3 Results

2.3.1. Identification of a cDNA clone recognised by antibody Pn14B7, sequencing, and sequence analysis

Immunological screening of a cDNA λZAPII expression library made from mRNA obtained from *P. nicotianae* zoospores yielded a positive clone. This clone was designated λZAPII-Pn14B7. The 3099 nucleotide long insert was sequenced and the nucleotide sequence analysed (Fig. 2.2). It became apparent that the cDNA clone was a chimerical clone consisting of two parts. The 5'-part is 2398 nucleotides long and is referred to as part 1 of the clone; the 3'-part is 701 nucleotides long and is referred to as part 2. In both cases, open reading frames could be found. The open reading frame of part 2 extended in the 5'-direction. Nucleotide-nucleotide searches (BLASTN) of both parts did not detect any significant homologies in the NCBI non-redundant database. The nucleotide sequence was translated into amino acid sequence. Part 1 is shown in Fig. 2.3 (frame +3), part 2 in Fig. 2.4 (frame +1). BLAST and pattern searches of the inferred amino acid sequence revealed that both parts are homologous to protein kinases.

Sequence analysis of part 1 of the cDNA clone λZAPII-Pn14B7

In translated searches of the protein database (BLASTX) and translated databases (TBLASTX), part 1 of the clone shows high homology to eukaryotic translation initiation factor 2-alpha kinases (see Appendices II and III). The inferred amino acid sequence between residues 481 and 667 of part 1 of the cDNA clone has some homology (61.3% aligned) to a protein kinase domain (pfam00069.6 pkinase; http://www.ncbi.nlm.nih.gov/Structure/cdd/ cddsrv.cgi?uid=pfam00069&version=v1.60) when searching the conserved domain database (RPS BLAST). The SMART database recognises also a protein kinase domain in the predicted protein. Between residues 207 and 666 a phosphotransferase domain with possible dual function as serine/threonine/ tyrosine kinase (SMART accession no. SM00221) is suggested.

GAATTCGGCACGAGGCTCAGTGGACAAGACGAACCCGTTCATTGGGTACT

TCCAAACGGGCACAGAAGAGAATGATGTGTTGGAGACGGTTTCGGGAGCA

GCTAGGGCCAAGATGAAGGCTCCAATGCCGCCGTCCCCAGCACCAGTGCG

CCGTGTGCCTATTCGAACGCAGAGCAGCGCGGCGCTTTCGTCCATGTCGT

CGTTCTACCCGTCAGCATTCTTCACGCCGCATAAGACAACGGAGTGGCTT

GGCTCGCAGACTAGCCGCCTCCCGTTCTTTAAGCAGAGAGATGCGAAGCA

ATCCACGCAGCAAAGACAGCAGGACGAGCAAAAGAGACGTACAGCGTTCC

TGGATTCGTTGAAACTGGAACCATCAGCGCTGGCTGTGCAGGCCGCAAAG

GATGAAAACCAGGAGGAACCGGTGCAAAAGCAGAAGCAGAGAGCTTCGTT
mgforw
CAAGGCGCACTGTTTCGATGCCGGCTGCTATGTCTACGAAGGCAGCGGTA

GCAGCACTGTCGCCTATCGTAACTTCATCAGAGGTGAAGGACGTTACTAC

ATCGAGCTTAACTCCTGATGGTACTTGCACGTCGAAATCGCGCTTTGAAC

AGGATTTTGAGGTCGTGGGCTCTCTAGGTGAAGGTGGTCAAGGCGCTGTG

TTCAAAGTACGAAGCAAGGTGGACGGGTGCTATTATGCTATCAAGAAGGT

GGTGCTGCCTCGCGCTACGGAGCTTGATGCTAACCGCGCGGAGAGCCAAG

CACTGCGTGAAGTTCGTTTGATGGCATCCATGGCCCCGCATCCCAACGTG

GTGCGCTACCATACAGCGTGGACTGAGGTGGATGCGTGCGTTTCTCACAA

GAGCTCGTCGATGGAGAGCGTTCGTGGCTCAGACGTGCCGTCGCTCGAAA

GTGACTGTAGTCCTGAAAGCCTTCAGCGACAGCAACTGGAACTGCTGGAC
mgforw2
GAGGAGGCCGAGCTGCGAAGCATGGAGACTGAAGACCAGTCGTTTTCGCT

TGAGTTTTCGTCAAATTCACTTAGTTTCGACGAGTATTCCACTCCTGGAT

TCACGTTTGAGGACGAAGGCGAAGAGGAGGACTCGGTGGGTTTGTGTGTG

GATGATGAGGCTGCGTTCGGACAACCTGGTGACCTGAAGAACGAGGTTCG

CGTAGCTCCTCCTGCTTCTCGCGCTGAACCGGTGGTCATGAAGTCTCAGG

TTATTCTGTACATTCAAATGGAACTGTGCGGGACGGCAGTAAACTCTGTG

CCTTCGACTCCCACTGCAGGTGGCCACCAGCCAATTCATCATATTCTGGA
mgforw3
CCAGCTGAAGTCGCCTCAACAAGATATTCAGCATTATAGCGAAGAAACTC

Fig. 2.2 Nucleotide sequence of the insert of cDNA clone λZAPII-Pn14B7. Poly-A tails are printed in bold. Part 2 of the clone is separated from part 1 by an asteric. Sequences of primers are underlined; the primer names are given below the relevant sequence. Nucleotide sequence continued next page.

ACTCGAACCTGGGAGCATGGCTCCGATCTTCGTTGGAGGAGCGATCAGCG

TGGTCTAACACCTCCGATATCCACCAAGAAGGGTTGAAGCTGTTTCTCAG

TGCAGTTCAGGGTGTGGCTCACATGCATTCCTACGGCGTGATCCACCGCG

ACTTGAAGCCGGACAACATATTCATCCATGGCGATCAAGCGAAGATTGGC

mgforw4

GACTTCGGTCTATCCAAGTCTGTTTTTACGGACAGCTCGTCCTATGGGGC

TGTGTCACCTCGTGAGCGGCTGCTGGAGTTGGGACTAAGCGATGGAGATC

ACACGACGGCACTCGGTACGTTCACATACGCGTCTCCTGAACAGCTAGGG

TACCGCTTTAGCAGCAGCAATGTGCTGAAGAACGCGGCGACTCGTCTGAA

AAGCGCCAAGTACTCGATCAAGTCGGATATCTTTGCTCTGGGGGTGATTT

TACTGGAGCTATGCTGCCCGTTTAGCACGATGATGGAGCGTTCGCAAGTG

TTGACAGGCGTGCGCCACGGTGTTGTGCCCCATAAGGCGCGACAACACTT

TCCAATGGAGATGGATCTGGTGTTGCGTATGACTTCAATTGACCCTGGTG

AGAGACCCACGAGTGAAGAAGTTTGCGAGCAGCTACGCAAGATAATGGCA

mgforw5

ACGTCAGGCACTGCAGTCACGCCAGCATCAGCTCTGGAGGAGTTGCGAGA

GCTGCAGGCGAAACTCGCAGCCGCTGTCCGCAAGGTACGTGATCGTTCTC

AAGCTACTCTTCAGCTCGAGGCTCTTGTCTCCGAGTTGAACGACAAGGTC

CAGAACGTGGCCATTGCTCTTGCCTAAGAAAATCGAGCATGAAGTCCACG

AAGGACATCGCAGCGTGGAAAAAAATCTGGAAACCCAACGAAAAAGCAAA

TAACGACGACAAAGCAGCGCGAGACAAGCAGCTTCCTCGATGAAGACAAG

CGACCTCGCTAATTAGTGGCTTTTGTGTGTGCGAAGTAAACCAATCTGCA

CCCACGAAATTGTGCGTGCACG**AAAAAAAAAAAAAAAAAAAAAAAAA***C

CTCGCCTCGTGCCGAATTCGGCACGAGGCAACCAGGGCAGCGTCCGTAAA

ATGATGGACGAACAAAATGTCGTCCCTGAGCACTTGGCTAAACAGATCTT

GAAGCAGACGCTGTCTGCACTGGAGTACTGCCACTCTATGGGTCAGGTAC

ACCGCGATGTCAAGGCCGAGAACGTTTTGCTCTCGGAGAACGAGGACGGC

TCGATTACTGCTAAACTTGCGGACTTTGGCCTCTCGGAGGAGCTGGAGCT

mgrev

GGCCAATCGCCGTCTCGAGACTATGTGTGGCACTCCGCAATATCTGAGTC

CCGAGCTGGTGTCGGGTCGCCTGCACGGCACACCGGCAGACATCTGGAGC

Fig. 2.2 Nucleotide sequence of the chimerical clone λZAPII-Pn14B7- continued. Nucleotide sequence continued next page.

```
ACAGGCATCCTCGCCTACATGATGCTGACTGGACTAGTGCCATTCGATGA

GGCCAAGAACGATGTGGAGCTGTTCAAGCTCATCAGTCTAGGCGCTGTGT

GGTACGATCAGCCGCAATGGGAATCGGTATCTGCAGAGGCTAAAAGCTTC

GTGCAGAGCATGCTGGACATTTCCCCGGAGTCGCGTCCTTCAGCAGCGGA

GCTCTTGAAGCACGAGTGGCTTCAAGACGCATGATGTAGCCATCTTTTGG

TAGGATGGCTACAGCATGCTTCATTATTAACTATTGTAGTGATTAGTATT

CAATTGAGTAGGTTTAACAAGTTTCACAGTTAAAAAAAAAAAAAAAAAAA
```

Fig. 2.2 Nucleotide sequence of the chimerical clone λZAPII-Pn14B7-continued

```
IRHEAQWTRR TRSLGTSKRA QKRMMCWRRF REQLGPR*RL QCRRPQHQCA
VCLFERRAAR RFRPCRRSTR QHSSRRIRQR SGLARRLAAS RSLSREMRSN
PRSKDSRTSK RDVQRSWIR* NWNHQRWLCR PQRMKTRRNR CKSRSRELRS
RRTVSMPAAM STKAAVAALS PIVTSSEVKD VTTSSLTPDG TCTSKSRFEQ
DFEVVGSLGE GGQGAVFKVR SKVDGCYYAI KKVVLPRATE LDANRAESQA
LREVRLMASM APHPNVVRYH TAWTEVDACV SHKSSSMESV RGSDVPSLES
DCSPESLQRQ QLELLDEEAE LRSMETEDQS FSLEFSSNSL SFDEYSTPGF
TFEDEGEEED SVGLCVDDEA AFGQPGDLKN EVRVAPPASR AEPVVMKSQV
ILYIQMELCG TAVNSVPSTP TAGGHQPIHH ILDQLKSPQQ DIQHYSEETH
SNLGAWLRSS LEERSAWSNT SDIHQEGLKL FLSAVQGVAH MHSYGVIHRD
LKPDNIFIHG DQAKIGDFGL SKSVFTDSSS YGAVSPRERL LELGLSDGDH
TTALGTFTYA SPEQLGYRFS SSNVLKNAAT RLKSAKYSIK SDIFALGVIL
LELCCPFSTM MERSQVLTGV RHGVVPHKAR QHFPMEMDLV LRMTSIDPGE
RPTSEEVCEQ LRKIMATSGT AVTPASALEE LRELQAKLAA AVRKVRDRSQ
ATLQLEALVS ELNDKVQNVA IALA*ENRA* SPRRTSQRGK KSGNPTKKQI
TTTKQRETSS FLDEDKRPR* LVAFVCAK*T NLHPRNCACT KKKKKKKK
```

Fig. 2.3 Inferred amino acid of part 1 of the chimerical clone λZAPII-Pn14B7 (frame +3). The sequence of the predicted protein is printed in bold.

The predicted molecular weight of part 1 (64.8 kDa) is significantly higher than the Pn14B7 antigen (approx. 40 kDa). The predicted isoelectric point is 5.55.

Sequence analysis of part 2 of the cDNA clone λZAPII-Pn14B7

In translated searches of the protein database and translated database, part 2 is homologous to doublecortin and/or calcium/calmodulin-dependent protein kinase subunits or similar proteins (see Appendices IV and V). RPS BLAST and SMART pattern searches recognised highest homology (68.8% alignment) to the catalytic domain of serine/threonine protein kinases (SMART accession no. SM00220; http://www.ncbi.nlm.nih.gov/Structure/ cdd/cddsrv.cgi?uid=smart00220&version=v1.60). The software program pI/Mw on the ExPasy Server predicted a pI of 4.57 and an approx. molecular weight of 19.76 kDa. These data are preliminary, as the gene sequence could extend in the 5'-direction.

PRLVPNSARG NQGSVRK**MMD EQNVVPEHLA KQILKQTLSA LEYCHSMGQV**
HRDVKAENVL LSENEDGSIT AKLADFGLSE ELELANRRLE TMCGTPQYLS
PELVSGRLHG TPADIWSTGI LAYMMLTGLV PFDEAKNDVE LFKLISLGAV
WYDQPQWESV SAEAKSFVQS MLDISPESRP SAAELLKHEW LQDA*CSHLL
VGWLQHASLL TIVVISIQLS RFNKFHS*KK KKK

Fig. 2.4 Inferred amino acid of part 2 of the chimerical clone λZAPII-Pn14B7 (frame +1). The sequence of the predicted protein is printed in bold.

2.3.2. Polyclonal serum against components present in zoospores of *Phytophthora nicotianae*

Polyclonal serum against *P. nicotianae* zoospores was obtained by injecting rabbits with either whole or ground zoospores that had been fixed with glutaraldehyde. Testing of the serum in indirect immunofluorescent assays revealed strong labelling over the entire cell (data not shown). The polyclonal serum was produced with the aim of using it to prepare a monospecific polyclonal serum that bound to the product of the cDNA identified by the Pn14B7 antibody. Since the cDNA clone identified in the screening process apparently did not represent the Pn14B7 antigen, monospecific polyclonal antibodies to this cDNA clone were not affinity purified.

2.3.3. N-terminal sequencing of immunopurified Pn14B7 antigen

Immunopurification using Pn14B7 yielded a polypeptide of approx. 40 kDa (data not shown). The size of the polypeptide corresponded well with the size recognised by Pn14B7 in immunoblots. N-terminal sequencing did not give any meaningful results suggesting that the N-terminus of the protein may be blocked.

2.3.4. Two dimensional gel electrophoresis of *Phytophthora nicotianae* zoospore proteins and immunoblotting with Pn14B7

P. nicotianae zoospore proteins were separated on a 2D gel (pH 3-10). Silver (Fig. 2.5) or Coomassie Brilliant Blue staining of the proteins showed that the amount of proteins that had been loaded was in the optimal range. The running conditions could be improved by increasing the isoelectric focussing time. On the immunoblot, monoclonal antibody Pn14B7 recognised a protein spot with a molecular weight of approx. 40 kDa at pH 3-3.5. The intensity of the immunostaining was too low to be photographically reproduced and a corresponding spot on the Silver or Coomassie stained gels could not be detected.

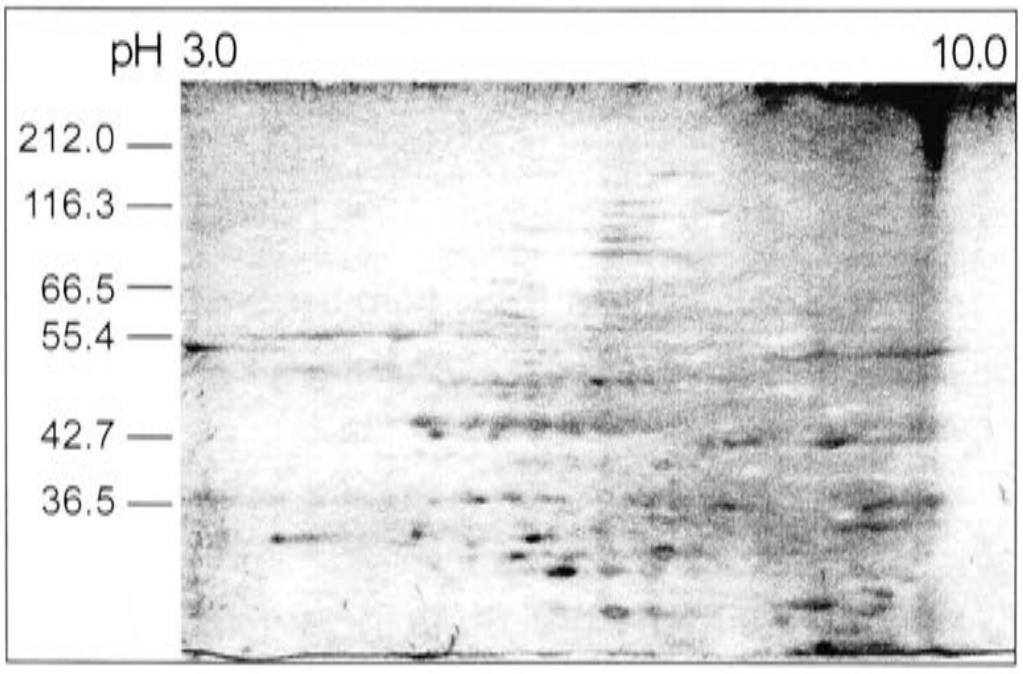

Fig. 2.5 Silver stained 2D SDS-PAGE of *P. nicotianae* zoospore proteins. Numbers on the left indicate positions of molecular weight markers in kDa.

2.4 Discussion

The main goal of the work described in this chapter was to obtain more information about the Pn14B7 antigen that composes or is associated with the tubular mastigonemes on the anterior flagellum of *P. nicotianae* zoospores. Two different approaches were followed. On the one hand, various procedures were used to purify the Pn14B7 antigen in order to obtain information on the amino acid sequence of this protein. On the other hand, Pn14B7 antibody was used to immunoscreen a cDNA expression library in order to clone the gene encoding the Pn14B7 protein. Unfortunately, neither approach was successful.

A number of different procedures were used to purify the Pn14B7 protein for amino acid sequencing, including immunoaffinity purification and 2D-gel electrophoresis. Sufficient Pn14B7 protein was obtained by immunoaffinity purification for the protein band to be excised from a Coomassie stained 1D gel, however, it was not possible to get N-terminal amino acid from this sample and it appeared that the N-terminus of the protein may be blocked. It might be possible to obtain internal amino acid sequence data but this would require considerably larger amounts of purified protein that would be extremely laborious and difficult to produce.

With regard to the second approach in which a cDNA library was screened with Pn14B7, the cDNA clone that was purified and sequenced contained two inserts, both encoding putative kinases. It is highly unlikely that either of the two inserts represents the true Pn14B7 antigen since both open reading frames encoded protein kinases, and it is difficult to envisage how a protein kinase could be associated with the tubular shaft of the mastigoneme on the outside of the flagellum. In addition, the open reading frame identified in part 1 of the clone codes for a protein with a predicted molecular weight that is higher than the molecular weight of the antigen (64.8 versus 40 kDa). Part 2 encodes a protein of lower molecular weight than the Pn14B7 antigen (19.67 versus 40 kDa) but it cannot be excluded that the open reading frame of part 2 could extend in the 5'-direction. If the complete part 2 protein were smaller than 40 kDa the antigen could be glycosylated yielding a glycoprotein of the expected

size. However, antibody screening, whether in cytochemical or biochemical assays, always has the possibility of producing non-specific binding results. Re-screening of the *P. nicotianae* zoospore cDNA library while using more stringent procedures to reduce non-specific binding could potentially lead to cloning of the Pn14B7 gene. However, given the considerations described below, it was decided not to continue with this approach.

A crucial factor in the ability to clone a gene of interest from a cDNA library is the abundance of transcripts encoding that gene in the mRNA used to construct the library. Before commencing this work, it was recognised that the *P. nicotianae* zoospore library may not be the ideal developmental stage for screening. Although small numbers of packets of mastigonemes occur in vegetative hyphae of *P. cinnamomi*, the abundance of mastigonemes increases substantially during sporulation (Cope and Hardham 1994). Thus, if this timing of mastigoneme synthesis also occurs in *P. nicotianae*, as is likely, it would be better to screen a cDNA library made from mRNA isolated during sporulation. Such a library was not available for *P. nicotianae*. A cDNA library constructed using mRNA isolated from sporulating *P. cinnamomi* hyphae was available, but as the mastigoneme-directed monoclonal antibodies were consistently species-specific, it was considered that it was not sensible to screen the *P. cinnamomi* library.

In addition to large-scale synthesis during sporulation, there was also indirect evidence that suggested that mastigonemes may be synthesised in zoospores. Mastigonemes on the flagella of living *P. cinnamomi* zoospores have been shown to detach after labelling with mastigoneme-specific antibodies (Cahill et al. 1996), suggesting they could be detached during normal zoospore swimming, especially in the soil where they are likely to frequently collide with soil particles. As *Phytophthora* zoospores can swim actively for days before encystment, it thus seems likely that they may be able to synthesise and replace damaged or detached mastigonemes. Such a phenomenon has been shown to occur for the non-tubular hairs in *C. reinhardtii* (Nakamura et al. 1996). Immunofluorescent labelling of *P. cinnamomi* or *P. nicotianae* zoospores with monoclonal antibodies that recognise the shaft of the tubular hairs has shown packets of tubular hairs stored within free-swimming zoospores (Cope and

Hardham 1994, Robold and Hardham 1998). Since the tubular hairs are important for the movement of zoospores it is likely that they can be produced and replaced in mature zoospores. Thus, the *P. nicotianae* zoospore cDNA library certainly has the potential of containing clones encoding the Pn14B7 antigen.

Chapter 3 Characterisation of the interaction of monoclonal antibodies raised against zoospore components of *Lagenidium giganteum* with *Phytophthora cinnamomi* zoospores

3.1 Introduction

During the early stages of my PhD project, Dr. J. Kerwin (University of Washington) made available a number of monoclonal antibodies that had been raised against *L. giganteum* zoospores components. *L. giganteum* is also a member of the Oomycetes, but one which infects insects rather than plants. It is used as a biocontrol agent against mosquitoes. Although initially classified within a separate order, the Lagenidiales (Barr 1983), recent studies have indicated a close phylogenetic relationship between the genus *Lagenidium* and other members of the order Peronosporales which includes *Phytophthora* and *Pythium* genera. These studies are based on comparisons of spore ultrastructure and biochemistry (Dick 2001), of the sequence of subunit 2 of the mitochondrial respiratory protein cytochrome c oxidase (Hudspeth et al. 2000), and of sequences of the nuclear large subunit ribosomal DNA (Petersen and Rosendahl 2000, Riethmüller et al. 2002).

3.1.1. Infection cycle of *Lagenidium giganteum*

Despite its very different hosts, the life cycle and mode of infection of *L. giganteum* bear high similarity to those of *Phytophthora* and *Pythium* species. As in these latter genera, the main infective stage is the asexual biflagellate zoospore, and successful colonisation has proved to be strongly dependent on the production of zoospores (e.g. Domnas et al. 1986). *L. giganteum* zoospores attach to and encyst on the host cuticle. After monopolar germination, the insect cuticle is penetrated and hyphae ramify through the host tissue. Upon depletion of nutrients after the death of the host, sporangia develop on aerial

parts of hyphae and zoospores are released (Kerwin et al. 1997). Under unfavourable environmental conditions the mycelium can switch to sexual reproduction and thick-walled oospores can be produced.

3.1.2. Structural aspects of *Lagenidium*

Ultrastructural studies of *Lagenidium* zoospores are relatively limited but have shown that the structure and organisation of components in *Lagenidium* spores are similar to those seen in *Phytophthora* and *Pythium* spores (Berbee and Kerwin 1993, Domnas et al. 1986, Gotelli 1974). The pear-shaped nucleus comes to a point near the centre of the longitudinal groove along the ventral surface of the zoospore. The two basal bodies lie adjacent to the point of the nucleus and the two flagella emerge from the basal bodies in the centre of the groove. The cytoplasm of the zoospores contains mitochondria, lipid and lamellate fingerprint vesicles (a store of carbohydrates). The cortical cytoplasm of the zoospores contains flattened peripheral cisternae, large peripheral vesicles that have electron-lucent contents, and small peripheral vesicles that contain homogeneous, moderately electron-dense contents. Thus, the main apparent difference between the zoospores of these three genera is the recognition of two categories of small peripheral vesicles in *Phytophthora* and *Pythium* (Cope et al. 1996, Hardham 1995) and only one in *Lagenidium*.

Unpublished studies by Dr. J. Kerwin indicated that the monoclonal antibodies he had raised reacted with vesicles in the peripheral cytoplasm of the *L. giganteum* zoospores. Given the evidence that the vesicular compartment of Oomycete zoospores is crucial for the pathogenesis of this group of organisms (see Chapter 1), it was of considerable interest to determine if the *L. giganteum* antibodies were interacting with components equivalent to any of the four types of peripheral vesicles (peripheral cisternae, large peripheral vesicles, dorsal vesicles, and ventral vesicles) in *Phytophthora* zoospores. Characterisation of the antibodies using *P. cinnamomi* spores also had the potential of determining if there were one or two categories of small peripheral vesicles in *Lagenidium*.

The aims of this part of my thesis research were to test the reaction of the available *L. giganteum* antibodies on *P. cinnamomi* zoospores in immunofluorescence assays and to determine, using indirect immunogold microscopy, the ultrastructural localisation of the antigen(s) labelled by antibodies positive in immunofluorescence assays. The *L. giganteum* antibodies were also tested on immunoblots and any positive reactions were compared to those obtained with monoclonal antibodies raised against *P. cinnamomi*.

3.2 Materials and Methods

3.2.1. Culturing of *Phytophthora cinnamomi* and production of zoospores

P. cinnamomi 6BR (H1000; DAR 52646; ATCC accession no. 200982) was subcultured as described in Hardham et al. (1991b). Agar plugs with mycelia were placed onto a single layer of miracloth on V8 agar. After 5 days incubation in the dark at 25°C, the miracloth discs were transferred into 250 mL Erlenmeyer flasks containing approx. 80 mL V8 broth and incubated overnight at 23°C on a shaker set to 120 rpm. The discs were rinsed four times with mineral salts solution (MSS; 10 mM $Ca(NO_3)_2$, 5 mM KNO_3, 4 mM $MgSO_4$, and 2 mL of a solution containing 10 mM $FeSO_4$ and 10 mM ethylenediamine-tetraacetic acid [EDTA] per L) and placed back onto the shaker. On the following day, zoospore release was induced by rinsing the discs with cold distilled water and incubating at 4°C for 12 min. Zoospores were released at 18°C on a light box over a period of 75–90 min.

3.2.2. Monoclonal antibodies

Hybridoma supernatants of monoclonal antibody producing cell lines no. 1, 2, 4A, 4B, 5A, 5B, 6, 7A, 8A, 8B, 9, 11, 12, 15A, 18, 19A, 19B, 20, 21A, 21B, 24, 25, 26A, 26B, 27, 28, 29, 30A, 32, 34A, 34B, 35, 36, 37, and 38 were provided by Dr. J. Kerwin. The antibodies had been tested on *L. giganteum* zoospores fixed with formaldehyde plus glutaraldehyde. Data on the labelling pattern of antibody 21B was not available. Antibodies 1 and 16 were negative on *L. giganteum* zoospores and antibodies 9, 26A, 32, 35, and 37 reacted with the plasma membrane. The remaining antibodies label small and large peripheral vesicles in zoospores of *L. giganteum* (Dr. J. Kerwin, personal communication).

3.2.3. Immunofluorescent labelling of *Phytophthora cinnamomi* zoospores

Indirect immunofluorescence labelling of *P. cinnamomi* zoospores was carried out as described in section 2.2.6 with the following modifications. Zoospores and cysts were fixed in either 4% formaldehyde or in 0.2% glutaraldehyde plus 4% formaldehyde in 50 mM PIPES buffer pH 7.0 for 30 min at RT. The fixed cells were incubated with 10 µL primary antibody at 37°C for 90 min. Hybridoma supernatants were used diluted 1:4 in PBS in the case of monoclonal antibodies 2, 18, 19B, 20, 27, and 32 and undiluted in all other cases. *P. cinnamomi* monoclonal antibody Cpw-4 (Hardham et al. 1994) hybridoma supernatant served as a positive control, and PBS served as negative control. Sheep F(ab')2 anti-mouse antibody conjugated to fluorescein isothiocyanate (SAM-FITC; Silenus, Melbourne, Aust.) was diluted 1:30 in PBS and used as a secondary antibody. In each well, 10 µL of secondary antibody were applied and incubated for 1 h. Photographs were taken using Kodak Tmax400 film (400 ASA).

3.2.4. Immunoblot

Proteins of freeze-dried zoospores were solubilised in 8 M urea on ice. The insoluble fraction of the cells was removed by centrifugation at 11000 x g for approx. 2 min at 4°C. The protein content of the supernatant was estimated using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) with BSA as a standard. The samples were diluted in an equal volume of 2 x SDS sample buffer (125 mM Tris-HCl pH 6.8 containing 20% (w/v) glycerol, 5% (v/v) SDS, 10% (v/v) 2-mercaptoethanol, and 0.00025% bromophenol blue, Laemmli 1970), boiled for 3 min, chilled on ice, and 150 µg crude protein per curtain gel separated using a Bio-Rad Minigel apparatus and 10% SDS-PAGE (Laemmli 1970) using 25 mM Tris, 200 mM glycine, and 1% SDS as an electrode buffer. Proteins were transferred overnight onto a PVDF membrane (Immobilon P®, Millipore Corp., Bedford, MA, USA) with the current limited to 0.4 mA and the voltage set to 25 kV. Non-specific binding was blocked by incubating the membrane in TBST containing 5% skim milk powder for 2 h. From this point on

the protocol for immunoblotting given in section 2.2.2 was carried out with the following modifications. The membrane was cut into strips and the strips were incubated individually for 1.5 h at RT in 50 µL hybridoma culture supernatant per strip on clean Nescofilm (Azwell Inc, Ozaka, Japan) in a humid chamber. Monoclonal antibody Lpv-1 (5 µg mL^{-1}, Gubler and Hardham 1988) was used as a positive control. Incubation in the secondary antibody SAM-AP diluted 1:10000 in TBST was for 45 min.

3.2.5. Post-embedding immunogold labelling

Ultrathin sections of *P. cinnamomi* zoospores embedded in Lowicryl K4M resin (kindly donated by Prof. A.R. Hardham) were cut on a Reichert Ultracut microtome with a Diatome diamond knife and collected onto gold grids coated with formvar. Non-specific binding sites were blocked by incubation on 15 µL droplets of PBSBG for 15 min. The grids were transferred into droplets of primary antibody, incubated for 1 h, and rinsed under a gentle stream of PBS containing 0.2% Tween20 (PBST, approx. 80 mL). PBSBG was used as a negative control, and *P. cinnamomi* monoclonal antibody Lpv-1 diluted in PBSBG to a concentration of 5 µg mL^{-1} served as a positive control. Goat anti-mouse antibody conjugated to gold particles with a diameter of 10 nm (GAM-Au10; British BioCell International) was used as a secondary antibody. It was diluted 1:20 in PBSBG and the sections incubated for 1 h. The grids were washed with approx. 80 mL PBST and finally with approx. 50 mL distilled water. Sections were stained with 2% uranyl acetate for 4 min, rinsed with distilled water, stained with lead citrate for 1 min, washed with distilled water, and left to air-dry. The sections were viewed at 120 kV on a Hitachi7100 transmission electron microscope.

3.3 Results

3.3.1. Immunofluorescence labelling of *Phytophthora cinnamomi* spores with anti-*Lagenidium* antibodies

Indirect immunofluorescence labelling of glutaraldehyde/formaldehyde fixed zoospores with *L. giganteum* monoclonal antibodies 2, 4A, 6, 8A, 8B, 15A, 18, 19A, 20, 24, 25, 26B, 27, 28, 29, and 34B was negative. As expected, the positive control Cpw-4 labelled the cyst surface strongly (Fig. 3.1A). On zoospores, labelling with Cpw-4 led to few small fluorescent spots on the zoospore surface (not shown). Monoclonal antibodies 4B, 5A, 5B, 11, 19B, 21B, 30A, 34A, 36, and 38 labelled the cyst surface, often very brightly (Fig. 3.1B). These antibodies also lead to labelling of few small spots on the surface of zoospores (Fig. 3.1C). Monoclonal antibodies 7A and 21A showed inconsistent labelling: some cells were labelled brightly, the water expulsion vacuole of others was labelled or the flagella of some zoospores were brightly fluorescent (data not shown).

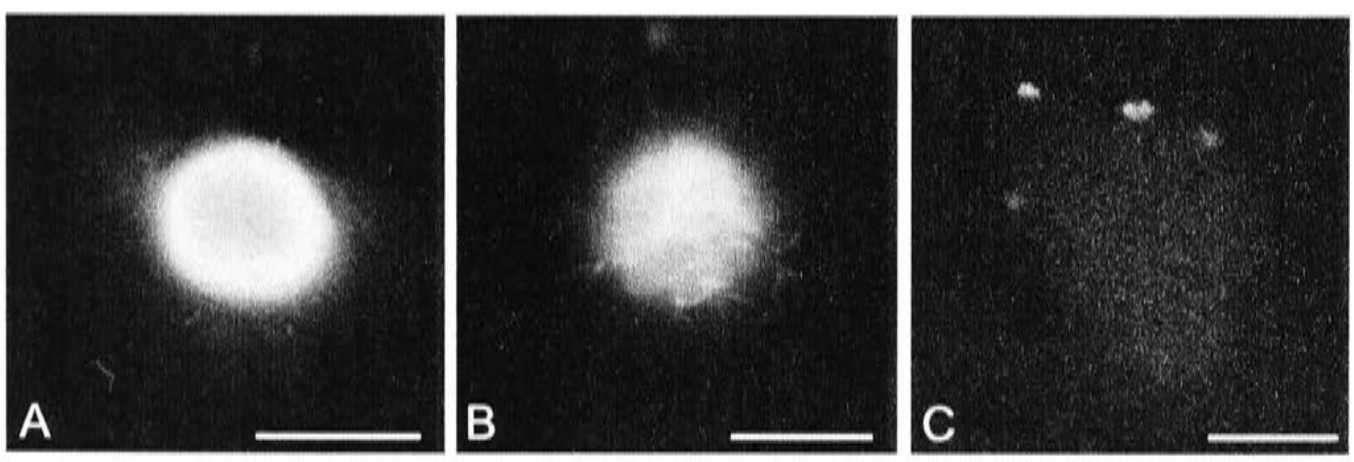

Fig. 3.1 Immunofluorescence micrographs of *P. cinnamomi* cells fixed with glutaraldehyde plus formaldehyde. The cyst in A was labelled with Cpw-4 (positive control). The cyst in B was labelled with *L. giganteum* antibody 4B. The cyst surface is strongly fluorescent. The zoospore in C was labelled with *L. giganteum* antibody 11. It shows four fluorescent spots on the cell surface. Size bars: 10 µm.

Immunofluorescence labelling of formaldehyde fixed zoospores with monoclonal antibodies 2, 4A, 8A, 8B, 19A, 20, 21A, 24, 25, 26B, 27, 28, 29, 34A or 34B was negative; monoclonal antibodies 5A, 7A, and19B lead to slightly enhanced background labelling. Monoclonal antibodies 4B, 5B, 6, 11, 15A, 18, 21B, 30A,

36, and 38 gave rise to tiny fluorescent spots over the entire surface of the zoospores (Fig.3.2A and B); they also labelled the surface of cysts, often brightly. Cpw-4 labelling lead to tiny spots on the surface of zoospores (Fig. 3.2C); the surface of cysts was also brightly labelled (data not shown).

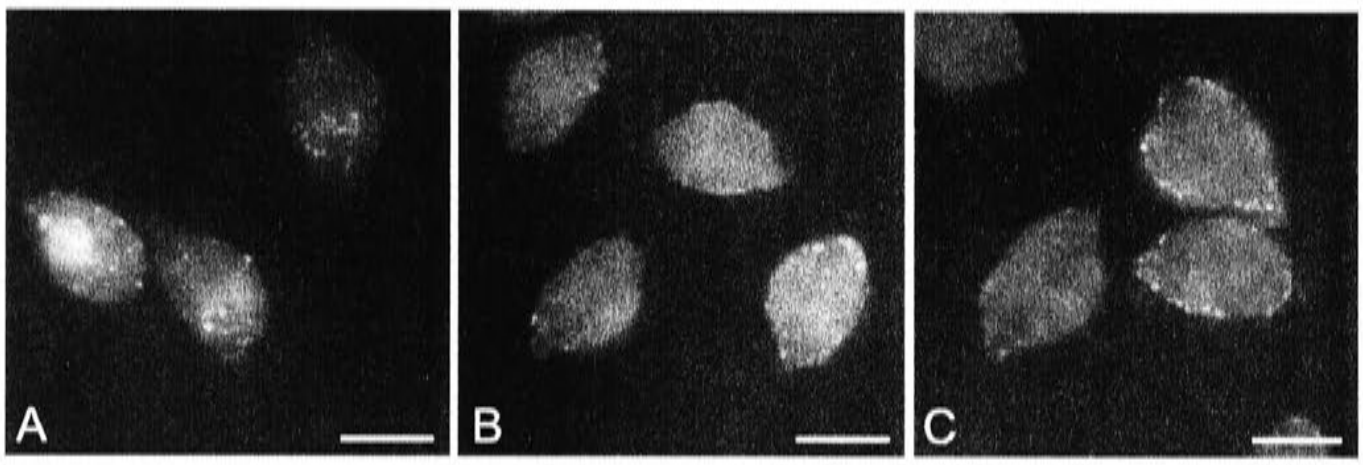

Fig. 3.2 Immunofluorescence micrographs of *P. cinnamomi* zoospores fixed with formaldehyde only. The zoospores in A were labelled with *L. giganteum* antibody 30A, and in B with *L. giganteum* antibody 36. In all zoospores, small weakly fluorescent spots are present. The zoospores in C were labelled with Cpw-4 (positive control). Size bars: 10 µm.

3.3.2. Ultrastructural localisation of zoospore antigens

Ultrathin sections of *P. cinnamomi* zoospores embedded in Lowicryl K4M resin were immunolabelled with *L. giganteum* antibodies that had been positive in indirect immunofluorescence assays. None of these monoclonal antibodies recognised its epitope in indirect immunogold assays (data not shown).

3.3.3. Biochemical characterisation of *Phytophthora cinnamomi* zoospore antigens

Crude protein extracts of *P. cinnamomi* zoospores were separated on a 10% SDS-PAGE, blotted onto a PVDF membrane, and immunolabelled with monoclonal antibodies 4B, 5A, 5B, 6, 7A,15A, 21A, 21B, 30A, 34A, 36 or 38. Monoclonal antibodies 4B, 5B, 30A, 36, and 38 recognised the same set of multiple high molecular weight polypeptides, three of these strongly (Fig. 3.3). These results were compared to immunoblots of protein extracts from *P. cinnamomi* zoospores labelled with monoclonal antibodies Lpv-1, Cpa-2, and Vsv-1 (compare Fig. 1.3). These antibodies label the contents of large peripheral vesicles, dorsal vesicles, and ventral vesicles, respectively. Lpv-1,

Cpa-2, and Vsv-1 labelled one to three high molecular weight polypeptides, as previously described (Gubler and Hardham 1988, Hardham and Gubler 1990).

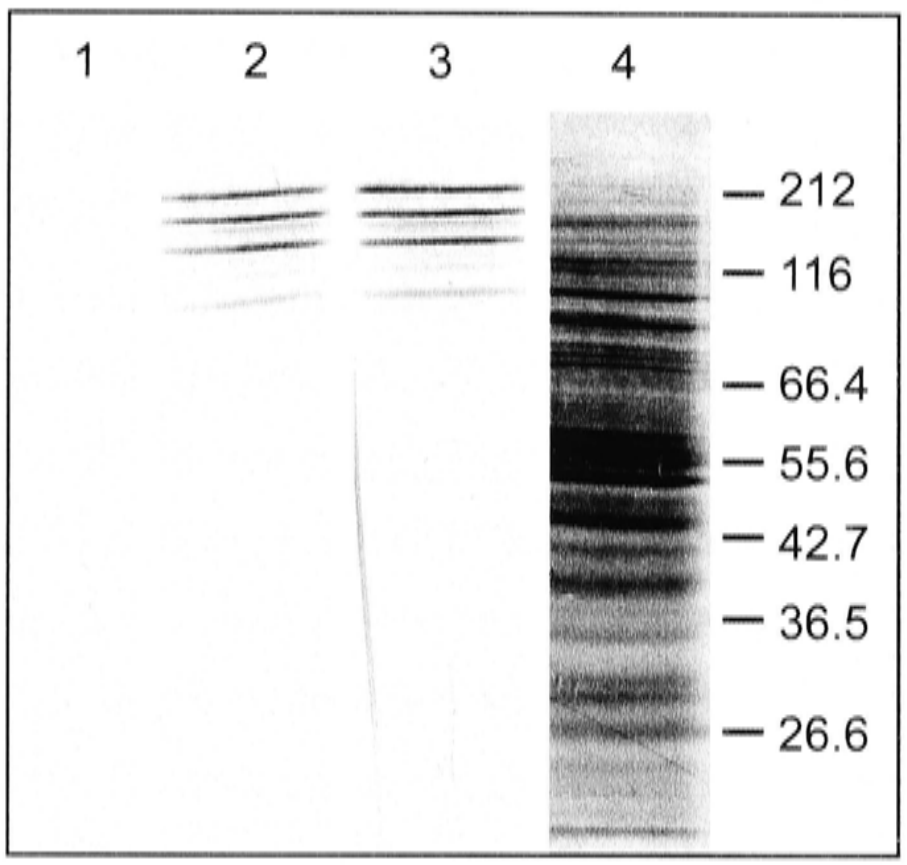

Fig. 3.3 Immunoblots (lane 1 – 3) and Coomassie stained (lane 4) extracts of *P. cinnamomi* zoospores separated by SDS-PAGE on a 10% gel. Lane 1 was incubated without primary antibody, lane 2 was incubated with *L. giganteum* antibody 4B, and lane 3 with *L. giganteum* antibody 5B. Antibodies 4B and 5B recognise multiple polypeptides; three polypeptides with an apparent molecular weight larger than 150 kDa react particularly strongly. Numbers on the right indicate positions of molecular weight markers in kDa.

3.4 Discussion

According to the information supplied by Dr. J. Kerwin, the monoclonal antibodies raised against *L. giganteum* react with vesicles located in the cortical cytoplasm of *L. giganteum* zoospores or with the plasma membrane. Antibodies 2, 4A, 8B, 12, 19A, 21A, 24, 25, 27, 28, 29, and 34B were described as labelling small vesicles; antibodies 8A, 19B, and 38 reacted with large vesicles; and antibodies 4B, 5A, 5B, 5C, 6, 7A, 7B, 11, 15A, 15B, 18, 20, 26B, 30A, 30B, 34A, and 36 recognised small and large peripheral vesicles; antibodies 9, 26A, 32, 35, and 37 label the plasma membrane (Dr. J. Kerwin, personal communication). Information on the labelling pattern of 21B was not available. Testing of these antibodies with *P. cinnamomi* zoospores and cysts showed that 13 of the 35 antibodies recognised antigens associated with the *P. cinnamomi* cells. All 13 antibodies gave the same pattern of labelling in immunofluorescence assays. Only five antibodies were positive on immunoblots reacting with the same set of polypeptides. The immunofluorescence assays showed binding to a few spots in glutaraldehyde/formaldehyde fixed zoospores, to numerous spots in formaldehyde fixed zoospores, and to the surface of cysts after both fixations. This distribution of antibody binding is consistent with the labelling of vesicles in the zoospore peripheral cytoplasm and their secretion during encystment.

The patterns of labelling of these antibodies were compared to those obtained with four *P. cinnamomi* monoclonal antibodies that react with different zoospore peripheral vesicles in order to obtain further information on the components being labelled in both *L. giganteum* and *P. cinnamomi*. As described and illustrated in Chapter 1, monoclonal antibodies Lpv-1, Cpa-2, and Vsv-1 react with the contents of large peripheral, dorsal, and ventral vesicles, respectively (Gubler and Hardham 1988, Hardham and Gubler 1990). In formaldehyde fixed zoospores, Lpv-1 labels large spots over the majority of the zoospore surface (Fig. 1.2A); it does not label the surface of cysts. Cpa-2 labels small spots distributed predominantly over the dorsal surface of zoospores and the entire

surface of cysts (Fig. 1.2B). Vsv-1 labels small spots that occur predominantly on the ventral surface, especially along the ridges of the groove (Fig. 1.2C). Binding to cysts is initially confined to the area corresponding to the zoospore ventral surface (Fig. 1.2D) but spreads to cover the entire cell surface as the cysts age. Cpw-4 was shown in the present study to label small spots over the surface of zoospores and the entire cyst surface in both fixations used.

Comparison of the labelling patterns of the *L. giganteum* antibodies with those of the four *P. cinnamomi* monoclonal antibodies clearly indicates that the *L. giganteum* antibodies are not reacting with the contents of the large peripheral, ventral or dorsal vesicles. The molecular weights of the polypeptides labelled are also different. However, the immunofluorescence labelling pattern shown by the *L. giganteum* antibodies is similar to that obtained with the *P. cinnamomi* antibody, Cpw-4. This antibody is part of a group of genus-specific antibodies that react with antigens associated with the peripheral cisternae and cell wall (Cpw antibodies; Hardham et al. 1994). Cpw-1, another *P. cinnamomi* Cpw antibody, reacts with a smear of polypeptides in immunoblots (Hyde et al. 1991). Cpw-1 binding is sensitive to pronase digestion of the antigen, indicating that the epitope recognised is proteinaceous in nature. Because none of the *L. giganteum* antibodies reacted with large peripheral, dorsal or ventral vesicles, they were not used any further in my PhD project.

Chapter 4 Production of monoclonal antibodies to components of *Phytophthora nicotianae* zoospores

4.1 Introduction

4.1.1. The function of antibodies

In mammals, the response to foreign molecules and organisms is highly refined. Components that trigger the immune response are present in the blood stream. These components are called antigens and there exists a wide variety of substances inducing immune responses. Upon presentation with an antigen, the body starts a cascade of events that, in most cases, leads to the elimination of the antigen. The cascade starts with the recognition of the antigen; the antigen is presented to specialised cells, and finally removed (Goding 1983, Harlow and Lane 1999). In the blood stream, antigens are carried to the spleen where they bind to lymphocyte receptors. In the beginning of a response this binding is often weak. In a complex sequence of events that involves the interaction of these lymphocytes with a different type of lymphocyte and the rearrangement of antibody-encoding gene segments a new antibody with high affinity to the antigen in question is made to measure. The lymphocyte producing the antibody is now terminally differentiated and continues synthesising exclusively this one antibody, the monoclonal antibody, for as long as it is needed. The antibody enters the blood stream and aids in the destruction of the antigen. Different lymphocytes can produce antibodies to different parts, or epitopes, of an antigenic molecule and this way, different antibodies can recognise a particular antigen. Mammals encounter many different antigens in their daily lives and a multitude of different antibodies circulate in their blood. Blood thus contains polyclonal serum.

4.1.2. The structure of antibodies

The key role of antibodies lies in their recognition of foreign molecules and in their communication of the presence of antigens to molecules or cells involved in the immune response. This dual function of antibodies is reflected in their structure (reviewed by Harlow and Lane 1999). Although the structure between the different classes of antibodies varies slightly, they are generally Y-shaped (Fig. 4.1). Each Y-shaped molecule is made up of four polypeptides, two identical heavy chains with an approximate molecular weight of 55 kDa, and two identical light chains of around 25 kDa. Five classes of antibodies (immunoglobulins [Ig]) are known: IgA, IgD, IgE, IgG, and IgM. The classes of antibodies are named after the type of heavy chain they contain. There are five types of heavy chains; α-, δ-, ε-, γ-, and μ-chains. The antibody classes also differ in the number of Y-shaped molecules in the mature protein. For example, antibodies of the IgM class contain μ-chains and consist of five Y-shaped molecules. IgG molecules contain γ-chains and have one Y-like molecule. Each class of antibody can have either κ- or λ-light chains.

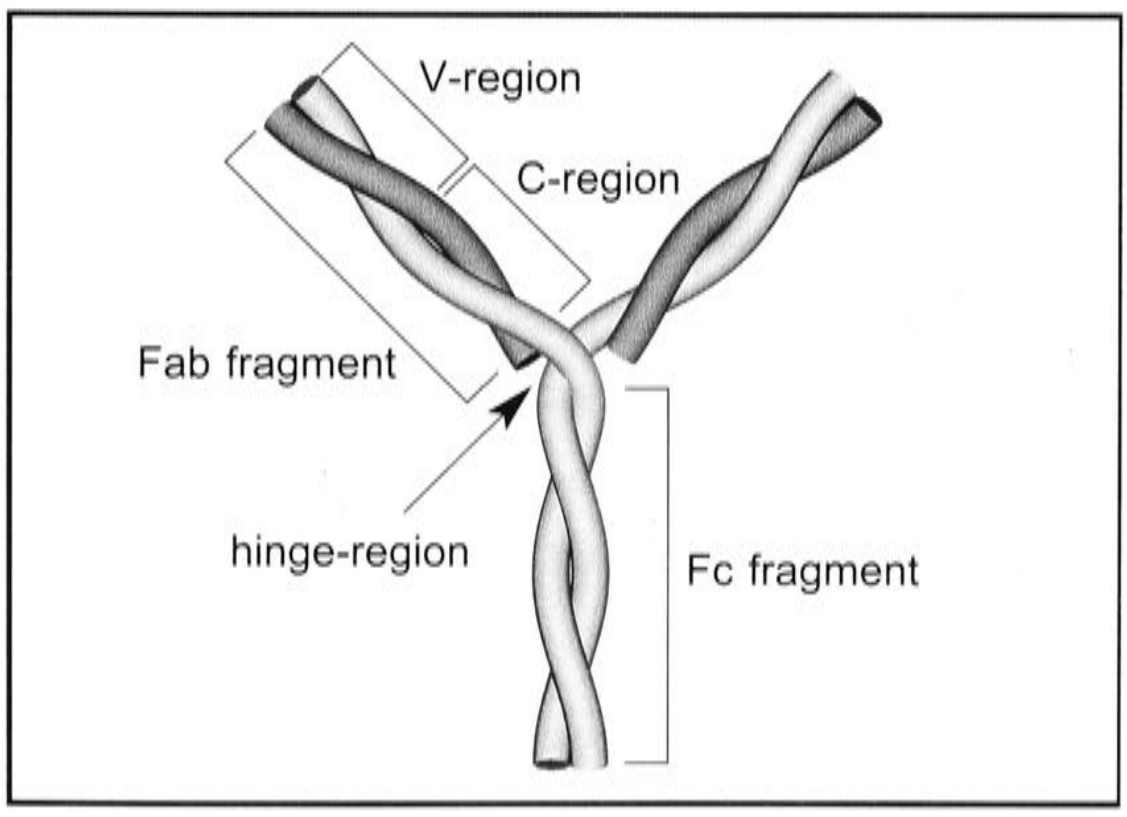

Fig. 4.1 Schematic representation of an IgG molecule. Light chains are printed in dark grey, heavy chains in light grey. Fc and Fab fragments are obtained after digestion of the protein with papain. The constant (C) and variable (V) region of the Fab fragment are shown. The hinge region allows flexibility of the antibody during interaction with its antigen.

At the base of each Y-shaped molecule, the two heavy chains are intertwined. They open up towards the arms, where each of the heavy chains interacts with one of the light chains. Disulfide bonds stabilise the interaction between both heavy chains as well as between heavy and light chains. The region in which the arms and base connect is flexible and called the hinge-region. The Y-shaped subunits are bivalent and flexibility of the antibody molecules in the hinge-region is thought to play a role in the sterical adaptability during antigen recognition. Both heavy and light chains have constant and variable regions. The amino-terminal half of the 220 amino acid long light chains is variable and is known as the V-region. The carboxy-terminal half is constant and known as C-region. Heavy chains also show a variable region of approx. 110 amino acids. They contain three constant regions of approx. 110 amino acids each. Antibodies of certain classes may have an additional constant region. The variable regions of light and heavy chains combine at the most distal part of the arms. This region represents the site of antigen recognition. In order to obtain as much variation in light and heavy chains as possible, the lymphocytes employ several strategies. Mutations within the regions of highest variability are generated; inaccuracies during genetic recombination take place; and the regions are encoded on multiple gene segments that can be combined in different ways.

Digestion of antibodies with the protease papain results in cleavage of the Y-shaped molecule into three fragments: the two arms and the base. The two arms have been named the Fab-fragments for *f*ragments with *a*ntibody *b*inding site. The fragment containing the base has been named the Fc-fragment for *f*ragment that *c*rystallises (reviewed by Harlow and Lane 1999). The Fc-fragment is involved in the binding of the antibody to other components involved in the immune response.

4.1.3. Polyclonal versus monoclonal antibodies as research tools

In scientific research, monoclonal or polyclonal antibodies can be employed. Polyclonal antibodies have been used in scientific research for a long time.

Advantages of polyclonal antibodies over monoclonal antibodies include the relative ease of their production: a mammal only needs to be immunised several times after which immunoreactive serum can be obtained. In many experiments, polyclonal antibodies are sufficient when tested for their specificity. They can be immunopurified using the antigen of interest and are in turn a valuable tool for affinity purification of their antigen as they can be used multiple times (Harlow and Lane 1999). However, when using polyclonal serum one has to be aware of the disadvantages. Polyclonal serum that has not been immunopurified almost certainly contains antibodies towards unrelated and often immunodominant antigens in a heterogeneous antigen mixture. This can be a drawback as specificity in antigen recognition is paramount. Additionally, only a limited supply of antibody is available. The amount available depends on the animal that has been used to generate the antibodies. For example, more polyclonal can be collected from a goat or rabbit than from a rat or mouse.

In 1975, the protocol for generation of monoclonal antibodies by means of permanent tissue culture was first described. Koehler and Milstein (1975) fused mouse myeloma and mouse spleen cells in order to obtain cultures of antibody secreting cells, the hybridoma cells. In this way an unlimited supply of specific antibody could be generated. The technique of Koehler and Milstein (1975) laid the groundwork for many exciting scientific discoveries. Monoclonal antibodies can be used in a variety of assays. They can be used to determine subcellular localisation of antigens, to purify an antigen, to find molecules that interact with the antigen, and to determine antigen concentration. However, a major problem still encountered is that of immunodominant antigens in mixtures of antigens used in the immunisation. For example, during the production of monoclonal antibodies against *P. cinnamomi* spore components, 31% of the monoclonal antibodies produced in a conventional fusion were directed towards an immunodominant cyst coat antigen (Hardham et al. 1986). The problem can be circumvented with various strategies. The preparation used for immunisation can be depleted of antigens other than the antigen of interest (Springer 1980) or the animals used for the immunisation are rendered immunotolerant to the immunodominant antigen by injecting them with these molecules neonatally (Weigle 1973). The latter approach has been used successfully by various researchers (e.g. Quintans and Quan 1983, Golumbeski and Dimond 1986,

Hardham et al. 1991b). Immunosuppressing agents like cyclophosphamide can also be used (Matthew and Paterson 1983). An excellent review on the production of monoclonal antibodies towards antigens which induce only a weak immune response is given by Riggott and Matthew (1996). An alternative to neonatal tolerisation or immunosuppression is a strategy known as co-immunisation (Fisher et al. 1982).

4.1.4. Co-immunisation and the production of specific monoclonal antibodies

There are many situations, in which it is desirable to obtain antibodies that are directed towards a molecule that cannot be purified from a mixture of many other molecules. If this mixture includes immunodominant antigens then the production of antibodies to the protein of interest is unlikely if following the conventional monoclonal antibody protocol. For example, antibodies that target proteins that occur only at certain stages of the life cycle of an organism may be required. But if molecules that occur throughout the life cycle are immunodominant, as is often the case (e.g. Barclay and Smith 1986), antibodies to the target proteins will not be obtained. In these situations, co-immunisation is a convenient way to minimise the production of antibodies that recognise common antigens and to obtain antibodies towards specific antigens. If, as in the above example, monoclonal antibodies specific for a certain developmental stage were sought, the immunisations would be as follows: first, an animal is injected with cells of the immunodominant unwanted developmental stage. Once a clear immune response can be detected, polyclonal serum is obtained from the first animal. A second animal is then immunised with a mixture of the polyclonal serum and cells of the developmental stage of interest. After several booster injections the animal can be killed and the spleen cells fused with myeloma cells. In many cases, antibodies specific for the stage of interest can be obtained and antibodies towards immunodominant antigens avoided (e.g. Barclay and Smith 1986; Murdoch et al. 1998, Pain et al. 1994).

4.1.5. Antibodies in *Phytophthora* research

Both polyclonal and monoclonal antibodies have been used to characterise *Phytophthora* components. Antibodies have been used extensively to gather data on a wide range of important aspects of asexual sporulation. For example, the subcellular localisations as well as biochemical data have been determined for tubulin, calmodulin, and centrin in *P. cinnamomi*. Tubulin is an approx. 50 kDa protein that constitutes one of the major components of the cytoskeleton. Using antibodies, the position of the flagellar roots and other important cytoskeletal components made of tubulin within the zoospores has been reported (Hardham 1987a). The localisation of the calcium-dependent signal-transducing protein calmodulin in both flagella of zoospores was shown using an anti-pea calmodulin antibody (Gubler et al. 1990). Strongest antibody labelling occurred in the paraxonemal swelling that is present at the base of the anterior flagellum. On immunoblots of crude zoospore extracts the monoclonal antibody recognises a polypeptide of approx. 16 kDa, a relative molecular weight that is comparable to the calmodulin homologue in *Achlya ambisexualis*, another Oomycete (Suryanarayana et al. 1985). In zoospores, anti-centrin polyclonal and monoclonal antibodies have been shown to label the 20 kDa protein centrin. The antigen is present in flagella, the basal body connector, and one of the microtubular roots, R1 (Harper et al. 1995). Labelling studies also demonstrated that during encystment the centrin component of the R1 root moves by approx. 90° with regard to the nucleus (Harper et al. 1995). This finding is important as in flagellate algal cells centrin has been suggested to play a role in a number of calcium-dependent processes that lead to the contraction of specialised subcellular structures. A similar function of centrin in *Phytophthora* can be proposed.

Antibodies were not only used for cytological and biochemical studies but in recent years also for the molecular cloning of their antigens in *P. cinnamomi*. For example, the genes encoding the contents of the large peripheral vesicles have been isolated through use of polyclonal and monoclonal antibodies (Marshall et al. 2001). β-tubulin has been cloned and sequenced after a commercial anti-β-tubulin antibody was used for immunoscreening of a *P. cinnamomi* cDNA library. The gene sequence gives support to the fact that

oomycetes are more closely related to algae and protists than to true fungi (Weerakoon et al. 1998).

The aims of this part of my thesis were to generate monoclonal antibodies recognising *P. nicotianae* zoospore components that are expressed exclusively during asexual sporulation with a focus on Vsv-, Cpa-, and Lpv-like immunofluorescent labelling patterns. Selected antibodies could then be used to characterise their antigen on an ultrastructural, biochemical and, eventually, also a molecular level.

4.2 Material and Methods

4.2.1. Culturing of oomycete species

Details of the isolation of the strains used are presented in Gabor et al. (1993).

Culturing of *Phytophthora nicotianae*

P. nicotianae strain H1111 was used. Subculturing and production of zoospores is described in section 2.2.1. For induction of encystment the following three approaches were employed: (i) the zoospore suspension was shaken hard in a glass measuring cylinder for approx. 30 s, (ii) 27% (v/v) cleared rye medium (6% organically grown rye kernels) containing 2% sucrose and 1 M mannitol was added to the zoospore suspension or (iii) a drop of zoospore suspension was placed onto a tobacco seedling on a microscope slide.

Vegetative hyphae were grown in still cultures in 20 mL V8 broth in Petri dishes for 5 days in the dark at 25°C. They were harvested by collecting them into a double layer of Kimwipes (Kimberly-Clark Australia Pty. Ltd.) and rinsed with distilled water.

Sporulating hyphae were obtained by rinsing vegetative hypha in MSS and incubating them overnight at 23°C in the light on a horizontal shaker.

Culturing of *Phytophthora cinnamomi*

P. cinnamomi strain H1000 was used and the subculturing and production of zoospores is described in section 3.2.1. Encystment of zoospores was induced by placing vials containing zoospore suspension against a vortex mixer for 30 s.

In all cases encystment was monitored using a Zeiss Axioplan microscope (Zeiss, Wetzlar, Germany). In general, encystment of a large proportion of the zoospores was seen after 10 min.

Culturing of *Pythium aphanidermatum*

Py. aphanidermatum strain H200 (DAR 60714) was used. It was maintained on V8 agar as described for the two *Phytophthora* species. For production of

zoospores, seven agar plugs containing hyphae were plated per 90 mm Petri dish containing V8 agar and incubated for 5 days at 25°C in the dark. After this time, 15 mL distilled water added and incubated overnight during which time zoospores were released.

4.2.2. Plant material

Tobacco (*Nicotiana tabacum*) or *Eucalyptus sieberi* seeds were surface sterilised with 5% sodium hypochlorite for 5 min, rinsed briefly with distilled water, disinfected in 70% (w/v) ethanol for 5 min, and rinsed for 5 min in distilled water. They were transferred onto 2% water agar in Petri dishes. Tobacco seeds were incubated for 10 days at 25°C in the dark, *E. sieberi* seeds for only 7 days.

4.2.3. Microsomal preparation

Microsomal preparations were made by Dr. H. Mitchell and Ms. V. MacLean based on the methods of Giannini et al. (1988).

Microsomal hyphae preparation

The hyphal microsomal preparation was obtained by vacuum infiltration of freshly harvested vegetative hyphae in 1 x osmoticum buffer (0.2 M sorbitol, 50 mM MES (2-[N-morpholino]ethanesulphonic acid)-KOH pH 6.5, 0.1% (w/v) polyvinyl-polypyrrolidone-40, 2 mM $MgSO_4$, 5 mM KCl, and 1 mM EDTA) on ice for 5 min, homogenisation with mortar and pestle on ice with grinding sand, and filtering through Kimwipes. The preparation was centrifuged at 13000 x g for 15 min and the supernatant was recentrifuged for 40 min at 100000 x g. The resulting microsomal preparation was resuspended in a solution containing 250 mM sucrose, 5 mM Tris/MES-KOH pH 7.0, 2 mM dithiotreitol, and 10% (w/v) glycerol. It was stored at -75°C until use.

Zoospore microsomal preparation

Zoospore suspensions were added to 10 x osmoticum buffer in a ratio of 10:1 at RT. The cells were immediately sonicated for 40 s (8 x 5 s bursts) to break the

plasma membrane. Lysed cells were transferred to precooled centrifuge tubes on ice, centrifuged at 6000 x g for 15 min, and the supernatant re-centrifuged at 85000 x g for 1 h. The pellet contained mitochondrial fragments and microsomal membranes and after mixing with the anti-hyphae serum it was used to immunise another BALB/c mouse. Samples of zoospore microsomal preparations were also embedded in 2% agarose in 100 mM PIPES buffer pH 7.0, osmicated, embedded, sectioned, and stained as in section 4.2.6 to determine what cellular components were present in the preparation used for the immunisation. Fixations and resin embedding were carried out by Prof. A.R. Hardham.

4.2.4. Production of monoclonal antibodies to *Phytophthora nicotianae* zoospore antigens

Immunisations, fusion of spleen cells, hybridoma cell culturing, and isotyping of antibodies were carried out by Ms. J. Elliot.

For the generation of monoclonal antibodies an approach known as co-immunisation was followed (Barclay and Smith 1986): a first BALB/c mouse was immunised with a microsomal preparation from *P. nicotianae* vegetative hyphae. After five booster immunisations, serum (referred to as anti-hyphae serum) was obtained and tested in immunofluorescence assays on vegetative hyphae as detailed in section 4.2.5. The anti-hyphae serum was then added to a microsomal preparation of zoospores and used to immunise a second BALB/c mouse. Hybridoma cells were produced and cultured according to standard protocols (Hardham et al. 1991b). Isotyping of the resulting monoclonal antibodies was carried out using the Mouse Monoclonal Antibody Isotyping Kit (Amersham Pharmacia Biotech).

4.2.5. Screening of supernatants of hybridoma cell lines for anti-zoospore antibodies

Hybridoma supernatants were screened in multiple rounds in immunofluorescence assays. Before cloning of cell lines, immunoblots were

employed as a second screening assay. Supernatants of 40 hybridoma cell lines were screened at least twice in indirect immunofluorescence assays and on immunoblots of zoospores/cysts, vegetative hyphae, and sporulating hyphae.

The procedures for indirect immunofluorescence assays of zoospores and immunoblots are detailed in sections 2.2.6 and 3.2.4, respectively.

Indirect immunofluorescence assays

For indirect immunofluorescence assays on sporulating hyphae, hyphae were harvested and either fixed in 4% formaldehyde in 50 mM PIPES buffer pH 7.0 and washed with 100 mM PIPES buffer pH 7.0 or used fresh. The hyphae were embedded in TissueTek embedding compound (Miles Elkhart, IN, USA) in plastic moulds, and plunge frozen in liquid nitrogen. The samples were stored at -20°C until use. Sections of approx. 12 µm were made on a Reichert-Jung 2800 Frigocut E cryotome at -20°C and collected on gelatine coated multiwell slides. The assay was continued as for the zoospores. In indirect immunofluorescence assays, hybridoma supernatants were used undiluted in all cases. Polyclonal serum (1:500 diluted in PBS) or Pn14B7 hybridoma supernatant served as a positive control; the hybridoma culture medium (Dulbecco's Modified Eagle Medium [ICN Biomedicals Inc., USA] containing 10% foetal calf serum, 4 mM glutamine, 10 µM β-mercaptoethanol, and 100 U Penicillin G and 10 µg Streptomycin sulphate mL^{-1}) and PBS served as negative controls.

To test the anti-hyphae serum in indirect immunofluorescence assays on vegetative hyphae, hyphae were grown and harvested as described in section 4.2.1 and fixed for 30 min in 4% formaldehyde in 50 mM PIPES buffer pH 7.0. They were rinsed in 50 mM PIPES pH 7.0 before embedding in TissueTek and plunge-freezing them in liquid nitrogen. The samples were stored, sectioned, and immunolabelled as for the sporulating hyphae.

Roots of *E. sieberi* were infected with *P. cinnamomi* by immersion of seedlings in a suspension of zoospores for 5 min for investigation of cysts and for 15 min for investigation of later stages of infection. The latter preparation was then incubated for 1-3 days at room temperature in distilled water. Infected plant

tissue was fixed for 2 h in 4% formaldehyde in 50 mM PIPES buffer pH 7.0. Embedding, cryosectioning, and immunolabelling were carried out as for the sporulating hyphae.

Immunoblot

For the immunoblots, between 20 and 200 µg of protein from zoospores, vegetative hyphae or sporulating hyphae were loaded per lane or up to 500 µg were loaded per curtain gel. Initially, 20 µg of protein were loaded per lane and 150 µg per curtain gel. In cases, where antibodies did not recognise their signal, the amount of protein loaded was increased to see if the antigen could be detected eventually. The membranes were cut into strips after blotting and the immunoblots continued as described before. For incubation in the primary antibody, the strips were either incubated individually in 40-60 µL hybridoma culture supernatant per strip on clean Nescofilm (Azwell Inc, Ozaka, Japan) in a humid chamber or in 1 mL hybridoma culture supernatant diluted in 1 mL TBST on a shaker.

4.2.6. Embedding for transmission electron microscopy and indirect immunogold labelling of zoospores and cysts

Pre-embedding labelling of *Phytophthora nicotianae* zoospores

Zoospores were fixed in 1% glutaraldehyde in 50 mM PIPES buffer pH 7.0 for 30 min. They were washed once with 100 mM PIPES buffer pH 7.0, once with PBS, and once with PBSBG. The samples were incubated at RT in 300 or 500 µL primary antibody on a shaker for 60 or 70 min. PBS was used as a negative control. Cells were washed twice in PBS before incubating in 120 or 150 µL GAM-Au10 diluted 1:15 in PBSBG. After rinsing the samples as described above, the zoospores were re-fixed in 1% glutaraldehyde in 100 mM PIPES buffer for 40 min, rinsed three times with PIPES buffer, embedded in 2% low-melting agarose (Sigma type VII) in 100 mM PIPES buffer, and stored overnight in buffer at 4°C. They were fixed for 1 h in 1% OsO_4 in 100 mM PIPES buffer, rinsed three times with buffer, and dehydrated in a graded acetone series (10, 30, 50, 70, and 90%; 30 min per step). The samples were

transferred into dry acetone and left overnight at 4°C after changing the acetone after 15 min. They were incubated with ethanol:Spurr's resin (Bio-Rad Laboratories, Hercules, CA, USA) mixtures in a ratio of 2:1, 1:1, and 1:2 (2-3 h per concentration) before transfer into pure resin. After 15-30 min the resin was changed. The samples were embedded in embedding capsules after infiltration for 24 h in pure resin. The resin was polymerised overnight at 60°C. Ultrathin sections were cut on a Reichert Ultracut microtome with a Diatome diamond knife, collected onto gold grids coated with formvar, and stained for 30 min with uranyl acetate and 10 min with lead citrate.

Embedding of cells or tissue for transmission electron microscopy and post-embedding labelling

Suspensions of *P. cinnamomi* zoospores or *P. nicotianae* zoospores or cysts were fixed in 1% glutaraldehyde in 100 mM PIPES buffer pH 7.0 for 30 min at RT by adding an equal volume of cell suspension to double strength fixative. After fixation, the samples were rinsed three times with 100 mM PIPES buffer, embedded in 2% low-melting agarose (Sigma type VII) in 100 mM PIPES buffer, and dehydrated in a graded ethanol series (10, 25, 50, 75, and 95%; 30 min per concentration) at 4°C. They were transferred into dry ethanol and left overnight after changing the ethanol once after 5-15 min. Resin infiltration was carried out on ice. For this, the samples were incubated with ethanol:Lowicryl K4M resin mixtures in a ratio of 2:1, 1:1, and 1:2 (2-3 h per concentration) before transfer into pure resin. The resin was changed after 15-30 min and again after 24 h. The samples were embedded in aluminium trays after infiltration for 48 or 72 h in pure resin. Polymerisation was achieved under UV light in a nitrogen-purged atmosphere. Prof. A.R. Hardham kindly donated zoospores of *Py. aphanidermatum* embedded in Lowicryl K4M resin.

Post-embedding immunogold labelling was carried out as described in section 3.2.5. PBSBG was used as a negative control, and Pn9G7 undiluted hybridoma supernatant (Robold and Hardham 1998) as a positive control on *P. nicotianae*. When *P. cinnamomi* was examined, either immunopurified Vsv-1 (20 µg mL^{-1}; Hardham and Gubler 1990) or Cpa-2 (5 µg mL^{-1}; Hardham and Gubler 1990) served as a positive control. The sections were incubated for 45 min in secondary antibody.

4.2.7. Pronase and periodate treatment of protein extracts and immunodot blot

Immunodot blots were carried out to determine if the epitopes of the monoclonal antibodies are sensitive to treatment with pronase and/or periodate, indicating that the recognised part of the molecule consists of protein or carbohydrate, respectively. The protocol used was a slight modification of that given in Robold and Hardham (1998). In 200 µL 6 M guanidine 5 µg of freeze dried zoospores were solubilised and left at RT for 20 min, 10 mL TBS were added, the sample left for 10 min on ice, and centrifuged at 11000 x g for 10 min at 6-8°C. The supernatant was kept on ice until it was used. A 0.45 µm nitrocellulose membrane (Hybond C, Amersham Pharmacia Biotech) was equilibrated in TBS, assembled into a dot blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA), and rinsed with TBS. Various dilutions of the sample were made in TBS, 200 µL applied per well, and incubated for 10 min at RT. After three washes in TBS for 5 min the samples were treated for 1 h with either PBS, 50 mM acetate buffer pH 4.5, 0.1 or 1.0 mg pronase mL^{-1} PBS or 10 or 20 mM sodium periodate in acetate buffer, and then washed three times for 5 min in either PBS or acetate buffer. The apparatus was disassembled, the membrane marked and cut into strips. Immunolabelling as described in section 4.2.5 was carried out using hybridoma culture supernatant. Lpv-1 (2 µg mL^{-1}, Gubler and Hardham 1988) served as a positive control, TBST as a negative control. The incubation time in primary and secondary antibody was 45 min.

4.2.8. Purification of mouse monoclonal antibodies

Between 0.5 and 1.0 L of supernatant from hybridoma cell lines Pn-Vsv-1, Pn-Vsv-2, Pn-Vsv-3, Pn-Vsv-4, Pn-Vsv-5, Pn-Vsv-6, and Pn-Vsv-8 were subjected to 45% ammonium sulphate precipitation at 6-8°C overnight. The precipitate containing the partially purified antibodies was collected by centrifugation at 17700 x g for 30 min at 4°C and the supernatant discarded. The pellet was dissolved in PBS (approx. 10% volume of the original volume), dialysed overnight at 6-8°C in a dialysis tube with a molecular weight cut off of 10 kDa against 20 mM sodium phosphate buffer pH 7.0, and filtered through a 0.45 µm

PVDF membrane (Millex HV, Millipore). A protein G column (HiTrap ProteinG column, Amersham Pharmacia Biotech) with 1 mL volume was equilibrated with 10 mL PBS, approx. 25 mL of the sample applied, the column rinsed with PBS as before, and the antibodies eluted with 7 mL 0.1 M glycine-HCl pH 2.7 in seven fractions into 25 µL 1 M Tris-HCl pH 9.2 per fraction. The column was rinsed as before and the purification continued with the remaining sample. Samples were collected during the course of the purification process, separated using a 10% SDS-PAGE, and stained with Coomassie Brilliant Blue to track the antibody during the purification. All fractions of the eluate containing antibody were pooled and the antibody concentrated to 1.0 - 5.0 mg mL^{-1} using an Ultrafree® 15 centrifugal filter device (Biomax 50 K NMWL membrane, Millipore). Purified antibodies were again tested in indirect immunofluorescence assays and immunoblots as described in section 4.2.5 and stored at 4 or -75°C until use.

4.3 Results

For the production of monoclonal antibodies, a co-immunisation procedure was followed (Barclay and Smith 1986). Mice were immunised with a microsomal preparation from vegetative hyphae of *P. nicotianae*. After several booster immunisations, polyclonal serum was obtained and a second set of mice was immunised with a microsomal preparation from *P. nicotianae* zoospores mixed with the serum. In the initial screening, hybridoma culture supernatants were tested in immunofluorescence assays on formaldehyde-fixed and on glutaraldehyde/formaldehyde-fixed zoospores and cysts of *P. nicotianae*. Before cloning, the positive hybridoma cell supernatants were screened on immunoblots of *P. nicotianae* proteins.

4.3.1. Components of the microsomal zoospore preparation used for immunisations

Microsomal preparations from zoospores were osmicated and embedded for transmission electron microscopy to determine the subcellular structures present in the material used for immunisations. Fig. 4.2 shows an electron micrograph of a microsomal preparation. The fractionation was incomplete as organelles that should have been removed during the low-speed spin, for example, nuclei and mitochondria, were present in the microsomal preparation. Fingerprint vesicles, large and small vesicles, and microsomes were represented in the preparation amongst other subcellular components.

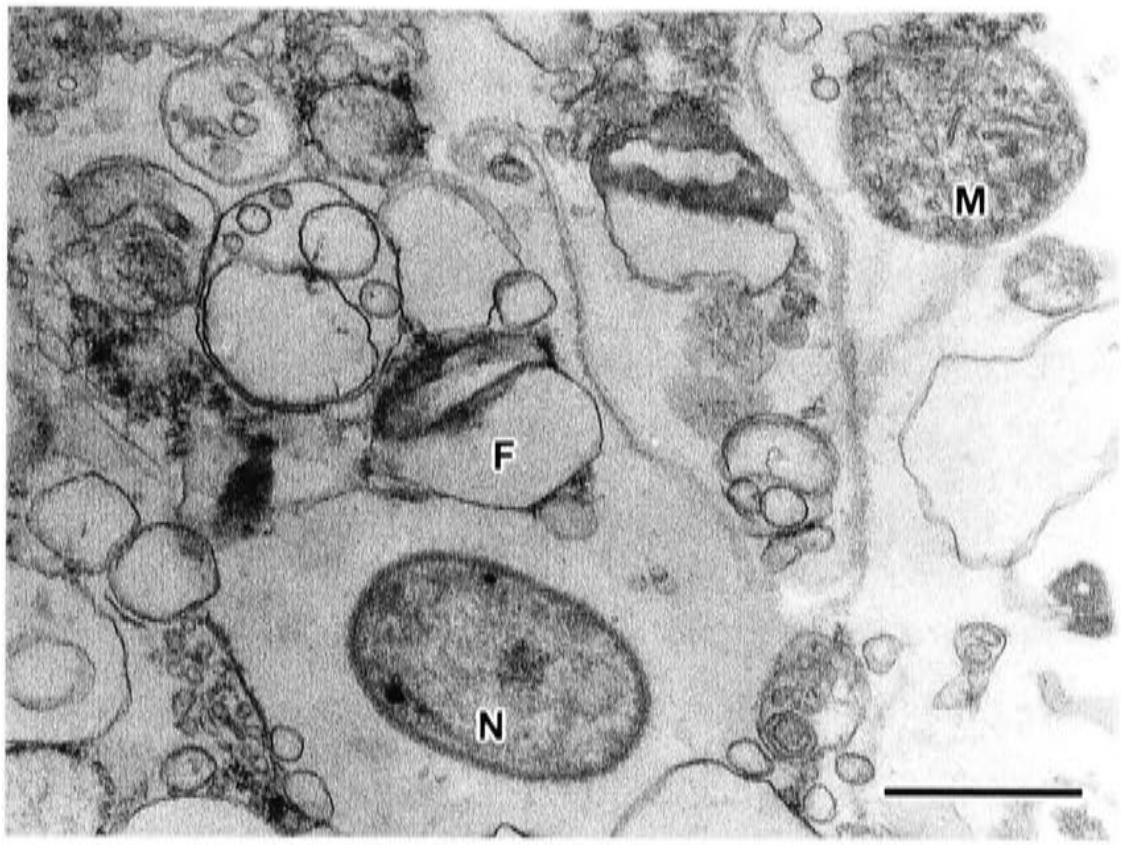

Fig. 4.2 Transmission electron micrograph of an osmicated *P. nicotianae* zoospore microsomal preparation. N: nucleus, F: fingerprint vesicle, M: mitochondrion; size bar: 1 µm.

4.3.2. New monoclonal antibodies to *Phytophthora nicotianae* zoospore components

Of the 364 cell lines that were screened, 90 remained positive after several rounds of screening in indirect immunofluorescence assays. The 40 hybridoma cell lines whose supernatants reacted most strongly in immunofluorescent assays were kept and supernatant testing on immunoblots revealed 29 cell lines that gave a positive result. Supernatants of the 40 cell lines were tested twice in indirect immunofluorescence assays and on immunoblots of zoospores and cysts, vegetative hyphae, and sporulating hyphae of *P. nicotianae*. Selected antibodies were tested in indirect immunogold assays and dotblots, and for their cross-reactivity with *P. cinnamomi* antigens. Ten hybridoma cell lines were cloned for use of the monoclonal antibodies in further experiments.

4.3.3. Localisation of zoospore and cyst antigens in indirect immunofluorescence assays

Immunofluorescent labelling of zoospores and cysts fixed with either formaldehyde only or with glutaraldehyde and formaldehyde was performed. Fixation with formaldehyde as the sole fixative allows the antibodies access to

the cytosol and its organelles due to poor preservation of the plasma membrane (Hardham 1985). The addition of glutaraldehyde to the fixative leads to good preservation of the plasma membrane. Thus, labelling of surface components only is possible on cells that are fixed with formaldehyde plus glutaraldehyde. Antibodies that reacted positively in immunofluorescence assays were divided into eight groups with regard to their fluorescence patterns. A summary of the labelling patterns of the antibodies is given in Table 4.1.

The largest group consisted of 15 monoclonal antibodies that reacted strongly with the entire surface of zoospores and cysts (Fig. 4.3) and with the exception of 10E7, also with the hyphal walls and sporangia of sporulating hyphae. Ten of these also reacted with a component in the hyphal cell wall of vegetative hyphae, nine of which gave rise to high background fluorescence on the microscope slide. The remaining five antibodies 7D9, 10E7, 11B2, 18E2, and 18G5 were negative on vegetative hyphae.

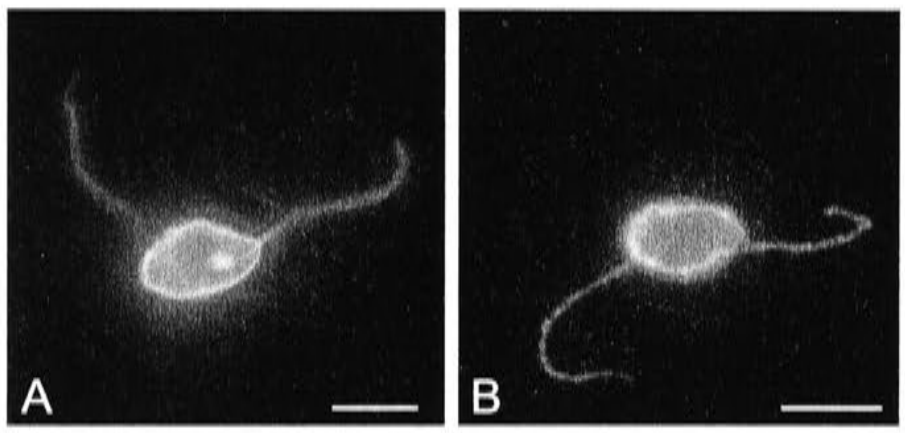

Fig. 4.3 Immunofluorescence micrographs of *P. nicotianae* zoospores fixed with glutaraldehyde plus formaldehyde. Cells in A were labelled with 10E7 and in B with 3B3. The surface of the zoospores is strongly fluorescent. Size bars: 10 µm.

Antibody 9F8 recognised a reticulum in formaldehyde fixed zoospores (data not shown). It did not label other developmental stages.

11G7 labelled the mastigonemes of zoospores fixed with a combination of glutaraldehyde and formaldehyde (Fig. 4.4). It was negative on all other developmental stages tested.

Table 4.1: Labelling patterns in indirect immunofluorescence assays

Monoclonal antibodies labelling the entire zoospore and cyst surface

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
1B4	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
2E5	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
3B3	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
7D8	surface	surface	hyphae	hyphae; sporangia
7D9	surface	surface	negative	spots inside hyphae; sporangia
10E7	surface	surface	negative	negative
11B2	surface	surface	negative	some spots inside hyphae; sporangia
12E2	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
15G8	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
16D4	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
16D7	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
18E2	surface	surface	negative	some spots inside hyphae; sporangia dull
18G5	surface	surface	negative	spots inside hyphae
19B10	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia
19C3	surface and background[1]	surface and background[1]	hyphae	hyphae; sporangia

[1] Antibody binds strongly to cells and the microscope slide

Table 4.1 – continued

Monoclonal antibodies labelling a reticulum within the *P. nicotianae* zoospores

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
9F8	reticulum	negative	negative	negative

Monoclonal antibodies labelling the mastigonemes

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
11G7	mastigonemes	negative	negative	negative

Monoclonal antibodies labelling the water expulsion vacuole

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
4D2	water expulsion vacuole	negative	negative	negative; no data for sporangia
5B9	water expulsion vacuole	negative	negative	negative; no data for sporangia
17B3	water expulsion vacuole	negative	negative	some labelling inside hyphae; water expulsion vacuole inside sporangia

Monoclonal antibodies labelling the anterior groove

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
3B4	anterior groove	negative	negative	some sporangia
6E9	anterior groove	negative	negative	spots inside hyphae; sporangia
9C2	anterior groove	negative	negative	negative

Table 4.1 – continued

Monoclonal antibodies labelling few vesicles in the zoospore cortex and the entire cyst surface

Antibody	Zoospore	Cyst	Vegetative hyphae	sporulating hyphae
10F6	a few spots in the cell cortex	surface	negative	some spots inside hyphae; sporangia dull
10F8	a few spots in the cell cortex	surface	negative	some spots inside hyphae; sporangia dull
12F4	a few spots in the cell cortex	surface	negative	some spots inside hyphae; sporangia dull
13F6	a few spots in the cell cortex	surface	negative	negative
14G2	a few spots in the cell cortex	surface	negative	some spots inside hyphae; sporangia dull

Monoclonal antibodies labelling the ventral vesicles

Antibody	Zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
Pn-Vsv-1	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-2	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-3	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-4	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-5	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-6	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-7	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-8	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-9	ventral vesicles	surface	negative	small vesicles inside hyphae and sporangia
Pn-Vsv-10	ventral vesicles	surface	hyphal wall; some spots in cytoplasm	small vesicles inside hyphae and sporangia

Table 4.1 – continued

Monoclonal antibodies labelling the large peripheral vesicles

Antibody	zoospore	Cyst	Vegetative hyphae	Sporulating hyphae
Pn-Lpv-1	large peripheral vesicles	negative	negative	large vesicles inside hyphae and sporangia
Pn-Lpv-2	large peripheral vesicles	negative	negative	large vesicles inside hyphae and sporangia

11G7 labelled part - probably the base - of the mastigonemes of zoospores fixed with a combination of glutaraldehyde and formaldehyde (Fig. 4.4). It was negative on all other developmental stages tested.

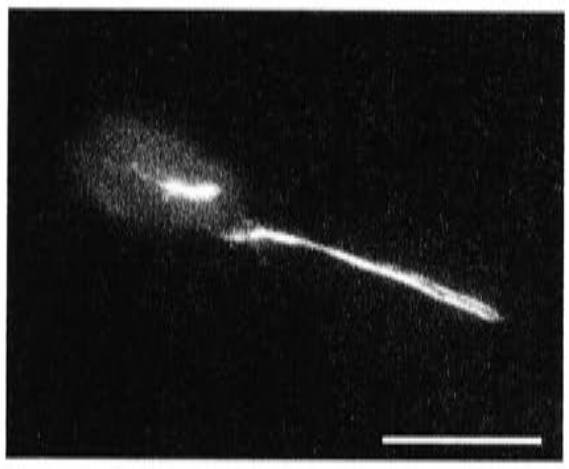

Fig. 4.4 Immunofluorescence micrographs of a *P. nicotianae* zoospore after fixation with glutaraldehyde plus formaldehyde and labelling with 11G7. The anterior flagellum is labelled by the antibody. Size bar: 10 µm.

Three monoclonal antibodies were directed towards the water expulsion vacuole of zoospores: 4D2, 5B9, and 17B3 (Fig. 4.5A); the latter also gave rise to fluorescent spots inside sporulating hyphae. It was the only antibody of this group that was tested on mature sporangia (Fig. 4.5B). It showed labelling of the water expulsion vacuole.

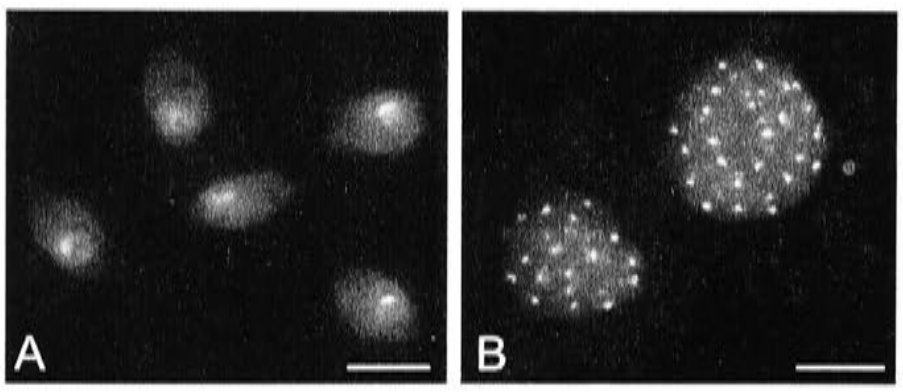

Fig. 4.5 Immunofluorescence micrographs after labelling with 17B3. The *P. nicotianae* zoospore in A was fixed with formaldehyde alone to allow the antibody access to the inside of the cell. Size bar: 10 µm. In B, a cryosection with two sporangia of *P. nicotianae* is shown. Size bar: 25 µm. The water expulsion vacuoles of free zoospores (A) and within the zoospores in mature sporangia (B) are labelled.

Three antibodies labelled the anterior and posterior part of the ventral groove of *P. nicotianae* zoospores: 3B4 (Fig. 4.6), 6E9, and 9C2. Sometimes, these antibodies reacted only with the anterior part of the groove. Only 6E9 also labelled sporulating hyphae and sporangia.

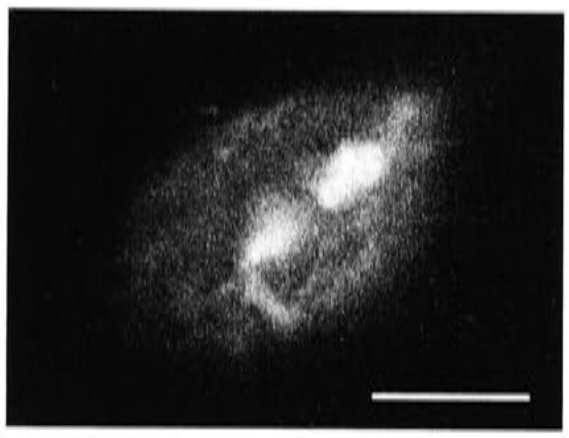

Fig. 4.6 Immunofluorescence micrograph of a *P. nicotianae* zoospore fixed with glutaraldehyde plus formaldehyde and labelled with 3B4. The regions of the groove on either side of the point of flagellar emergence are labelled. Size bar: 5 µm.

Antibodies 10F6, 10F8, 12F4, 13F6, and 14G2 led to strong surface labelling of cysts in both fixation protocols (Fig.4.7A, C, and D). When formaldehyde fixed zoospores were tested, most of them showed several strongly fluorescent spots and a varying number of spots with weaker fluorescence in the cell periphery (Fig. 4.7A). Addition of glutaraldehyde to the fixative usually abolished zoospore labelling. However, sometimes, fluorescent spots were still present in the zoospores (Fig. 4.7B).

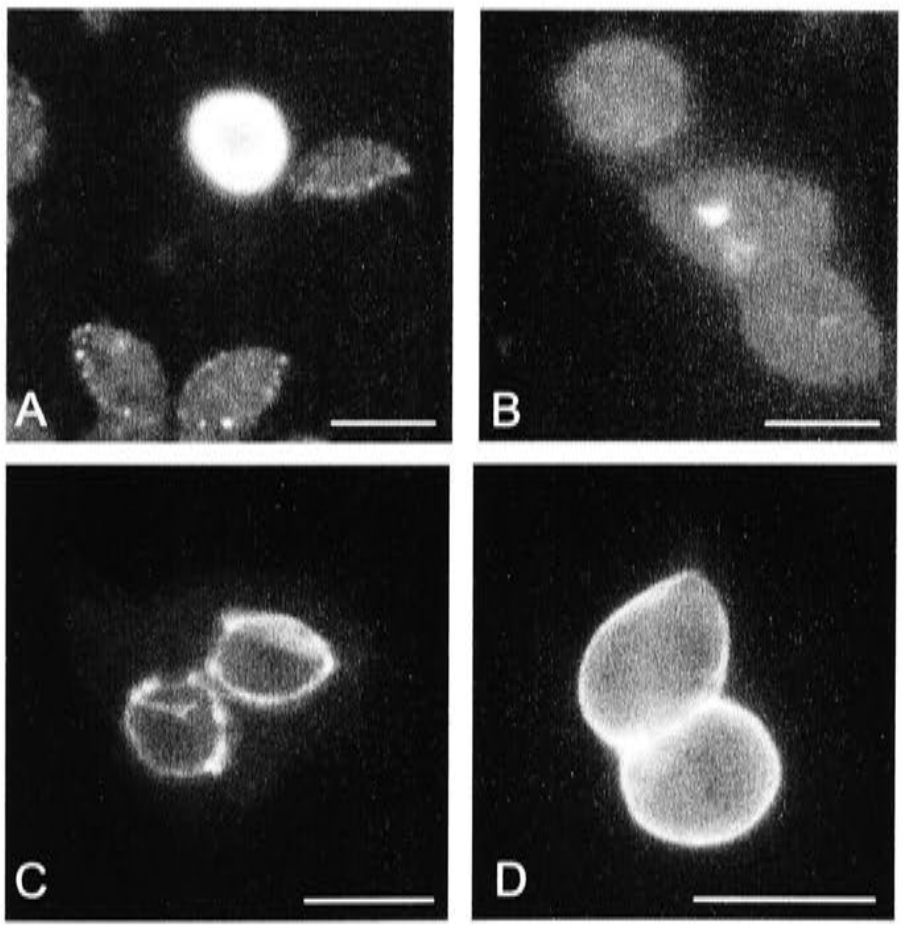

Fig. 4.7 Immunofluorescence micrographs of zoospores and cysts of *P. nicotianae*. Cells in A were fixed with formaldehyde alone and labelled with 12F4. The zoospores show peripheral punctate labelling; the cyst surface is labelled brightly. Cells in B, C, and D were fixed with glutaraldehyde plus formaldehyde. In B, zoospores labelled with 12F4 rarely show fluorescent patches. The encysting zoospores in C were labelled with 10F6, the cysts in D with 13F6. Figures C and D show that the immunofluorescent pattern is uneven at first and becomes smoother as encystment progresses. Size bars: 10 µm.

Ten antibodies labelled small peripheral vesicles in the same fashion as antibody Vsv-1 on *P. cinnamomi* sporulating hyphae, zoospores, and cysts (Hardham and Gubler 1990). Monoclonal antibody Vsv-1 recognises ventral surface vesicles in *P. cinnamomi*. The antibodies labelling the ventral vesicles that were obtained in this study are referred to as Vsv antibodies throughout this thesis and were named Pn-Vsv-1 to Pn-Vsv-10. They labelled small fluorescent spots in the cell cortex of formaldehyde fixed zoospores (Fig. 4.8A), and surface labelling could be seen in cysts fixed using either of the two fixation protocols (Figs. 4.8B and C). The surface labelling of cysts showed wide variation. On young cysts where the groove is still visible and the cell shape is not yet round, fluorescence in the form of patches on the ventral side of the cell could be detected. On fully encysted cells that had rounded up completely, bright fluorescence all over the cell surface indicated a more or less even distribution of the antigen(s) over the cells. The labelling patterns obtained with antibodies Pn-Vsv-1 to Pn-Vsv-10 were identical. The spatial distribution of the small peripheral vesicles containing their antigen(s) was neither clearly dorsal nor clearly ventral, although the fluorescent spots appeared mainly clustered along the central groove of the zoospores. Nevertheless, some of them were also present in other regions of the cell cortex of *P. nicotianae* zoospores.

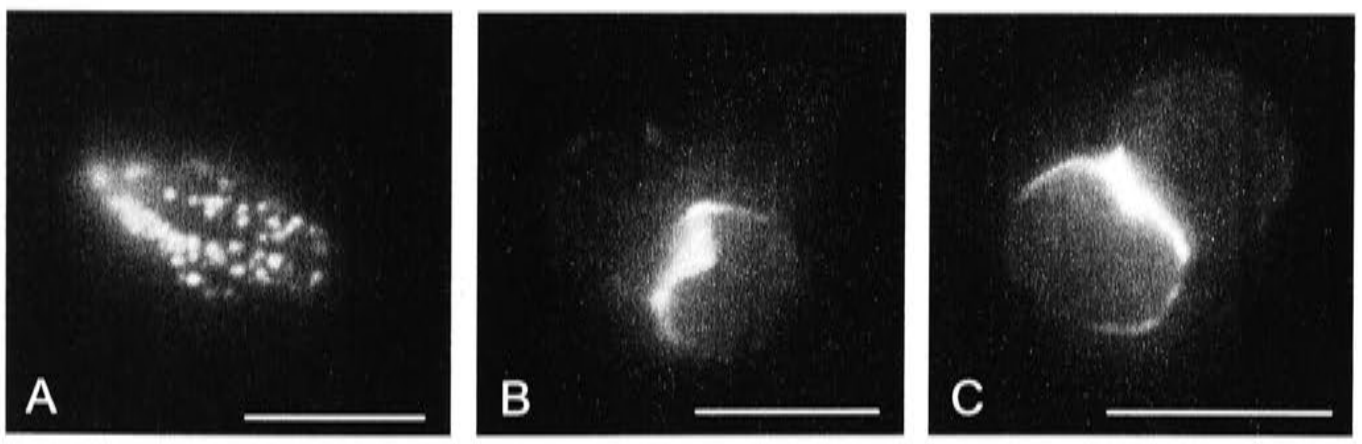

Fig. 4.8 Immunofluorescence micrographs of *P. nicotianae* zoospores and cysts labelled with Vsv antibodies. In A, the zoospore was fixed with formaldehyde alone and labelled with Pn-Vsv-1. The ventral vesicles occur mainly underneath the ridges of the groove of the zoospore and are labelled. In B and C, glutaraldehyde was added to the fixative, so only surface labelling was possible. The cyst in B is young and the region that had been the groove region is brightly labelled by antibody Pn-Vsv-3. On the cyst on the left in figure C, the material labelled with Pn-Vsv-2 has started to spread over the cyst surface wereas the contents of the ventral vesicles are still confined to the groove region on the cyst on the right. Size bars: 10 µm.

Two antibodies displayed the same labelling pattern as that observed on *P. cinnamomi* zoospores labelled with monoclonal antibodies targeting the large peripheral vesicles. The antibodies are referred to as Lpv antibodies Pn-Lpv-1 and Pn-Lpv-2. In *P. nicotianae* zoospores fixed with formaldehyde, fluorescence in the form of numerous large spots within the cytosol could be observed after labelling with Pn-Lpv-1 (Fig. 4.9A) or Pn-Lpv-2. Cells fixed with formaldehyde plus glutaraldehyde showed some large spots when tested with these monoclonal antibodies (Fig. 4.9B). Pn-Lpv-1 was an IgM isotype, Pn-Lpv-2 an IgG_3 isotype.

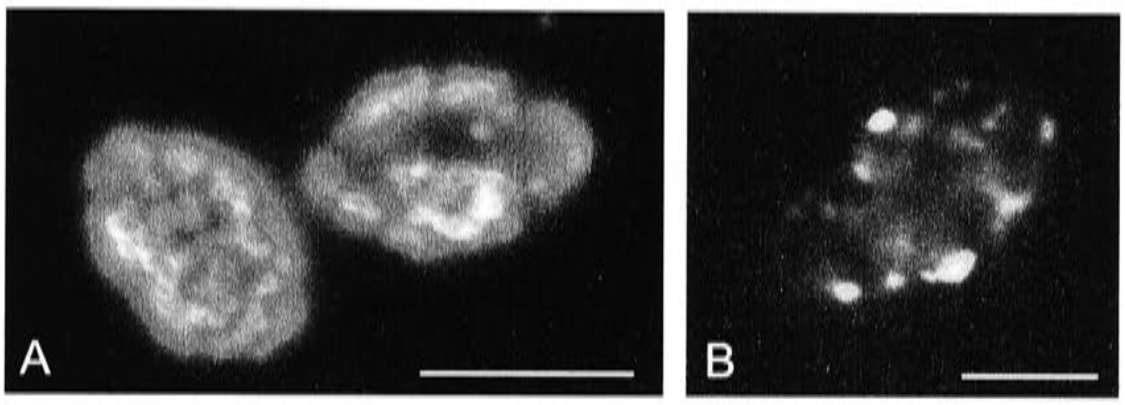

Fig. 4.9 Immunofluorescence micrographs of *P. nicotianae* zoospores labelled with Pn-Lpv-1. The cell in A was fixed in formaldehyde alone and many large vesicles are strongly labelled; the cell in B was fixed with formaldehyde plus glutaraldehyde and only a few large vesicles are fluorescent. Size bar in A: 10 µm, in B: 5 µm.

4.3.4. Crossreactivity of monoclonal antibodies to *Phytophthora cinnamomi* components in indirect immunofluorescence assays

For indirect immunofluorescent labelling, zoospores and cysts of *P. cinnamomi* were fixed with either formaldehyde or with glutaradehyde plus formaldehyde. For infected *E. sieberi* root tissue the former fixative was used. All Vsv antibodies with the exception of Pn-Vsv-10, cross-reacted with components in *P. cinnamomi* zoospores (Fig. 4.10A/B). They showed the same pattern of fluorescence on *P. cinnamomi* zoospores and cysts in both fixation procedures as on *P. nicotianae* cells. Only monoclonal antibody Pn-Vsv-3 was tested on tissue of *E. sieberi* infected with *P. cinnamomi*. Cysts on *E. sieberi* roots showed a patch of bright fluorescence between host root and pathogen after indirect immunofluorescence labelling with Pn-Vsv-3 (Fig. 4.10C/D).

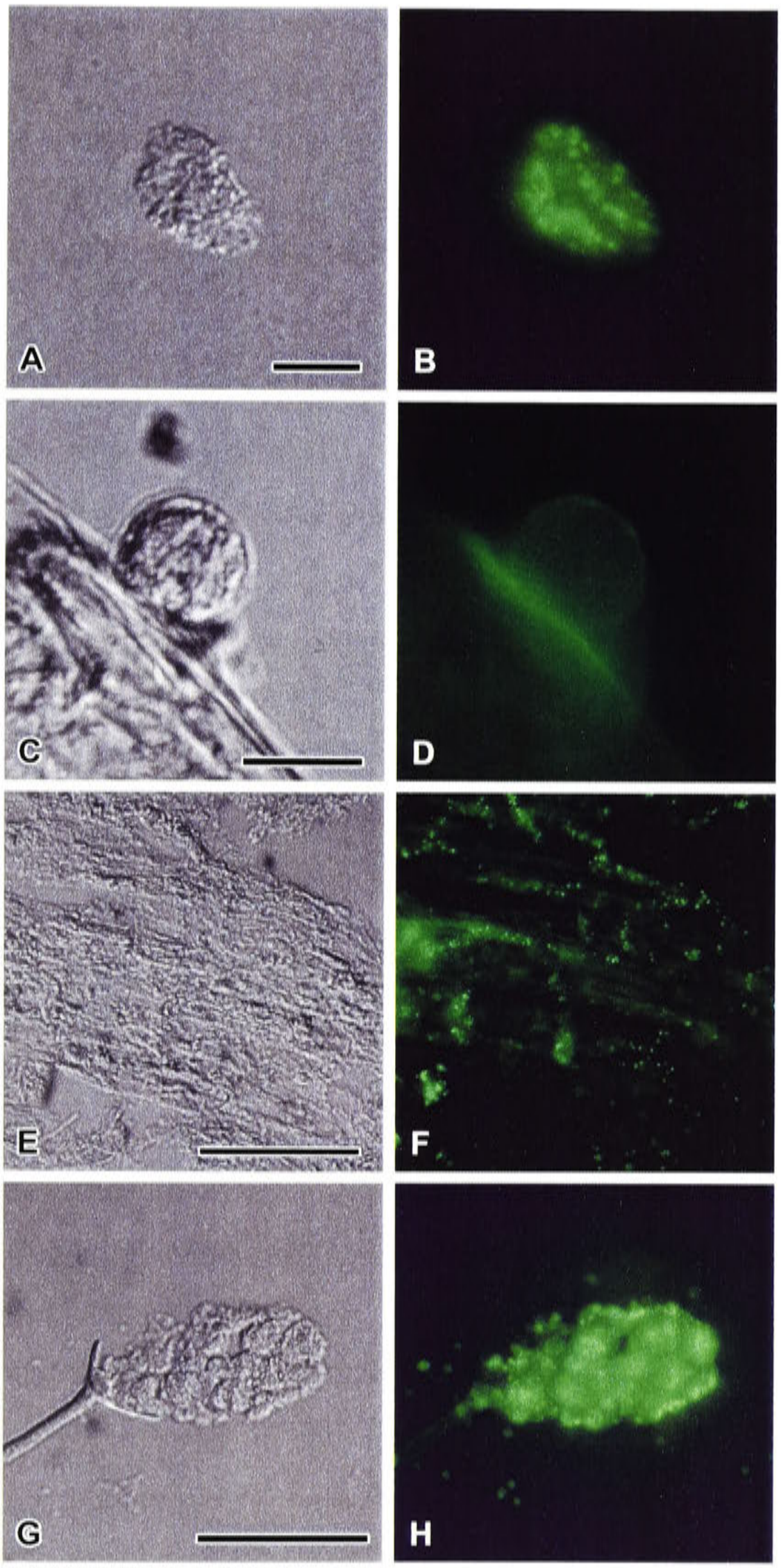

Fig. 4.10 Micrographs of *P. cinnamomi* infecting roots of *E. sieberi*. Photographs A, C, E, and G are taken with differential interference contrast optics, photographs B, D F, and H are taken with FITC filters. Figures A and B: zoospore; figures C and D: cyst adhering to a host root; figures E and F: infected root tissue 3 days after inoculation; and figures G and H: sporangium. Antibody Pn-Vsv-3 was used for immunolabelling. The putative adhesive stored in the ventral vesicles (B) is released during encystment to form a pad between cyst and host root (D). During preparation for sporulation, the ventral vesicles are formed within the hyphae (F) and are transported into the forming sporangia (H). Size bars in A and C 10 µm, in E 25 µm, and in G 50 µm.

When tested on *E. sieberi* roots infected with *P. cinnamomi* for 3 days before fixation and embedding, vesicles inside the hyphae as well as inside the developing sporangia were labelled (Fig. 4.10E/F). In mature sporangia peripheral vesicles inside the developing or developed zoospores reacted strongly with the antibody (Fig. 4.10G/H).

Pn-Lpv-1 and Pn-Lpv-2 labelled large peripheral vesicles in formaldehyde fixed *P. cinnamomi* zoospores (Fig. 4.11A). When glutaraldehyde was added to the fixative, only a few large peripheral vesicles were labelled (Fig. 4.11B).

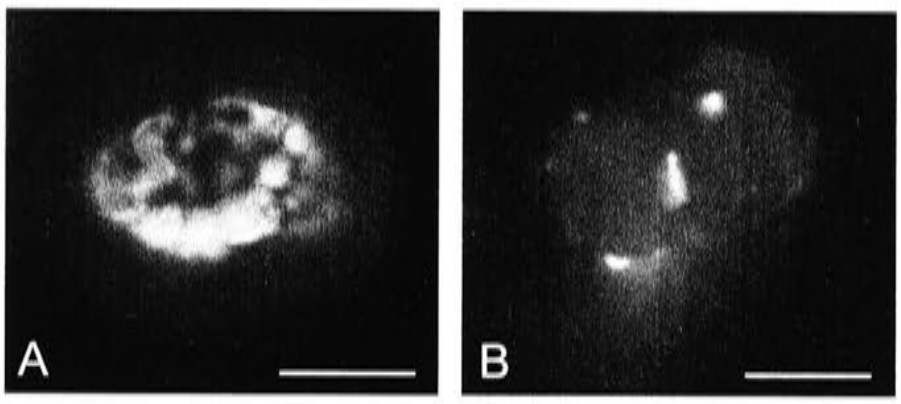

Fig. 4.11 Immunofluorescence micrographs of *P. cinnamomi* zoospores labelled with Pn-Lpv-1. The cell in A was fixed in formaldehyde alone; the cell in B was fixed with formaldehyde plus glutaraldehyde. The observed labelling pattern is identical to the one observed for Lpv-1. Size bar: 10 µm.

4.3.5. Ultrastructural localisation of vesicle antigens in *Phytophthora nicotianae* and *Phytophthora cinnamomi*

The monoclonal antibodies towards the cyst surface and a few peripheral vesicles in zoospores were tested twice in post-embedding indirect immunogold labelling of *P. nicotianae* zoospores. They failed to recognise their antigen.

Post-embedding immunogold labelling of sections of *P. nicotianae* zoospores with monoclonal antibodies Pn-Vsv-1 to Pn-Vsv-8 lead to labelling of some of the small peripheral vesicles (Fig. 4.12). These vesicles were less electron-dense than the large peripheral vesicles and typically showed an electron-lucent halo. In many cases gold particles were confined to this halo. Consistent with the immunofluorescence assay, the population of small vesicles containing the Vsv antigen(s) was mainly present underneath the ridges of the ventral groove. However, as seen in the immunofluorescence assay, some small vesicles in the

dorsal cell cortex were labelled. When monoclonal antibodies Pn-Vsv-9 or Pn-Vsv-10 were used, no specific labelling could be observed. Cysts of *P. nicotianae* were not labelled by the antibodies. Pre-embedding labelling of *P. nicotianae* zoospores with the Vsv antibodies was negative.

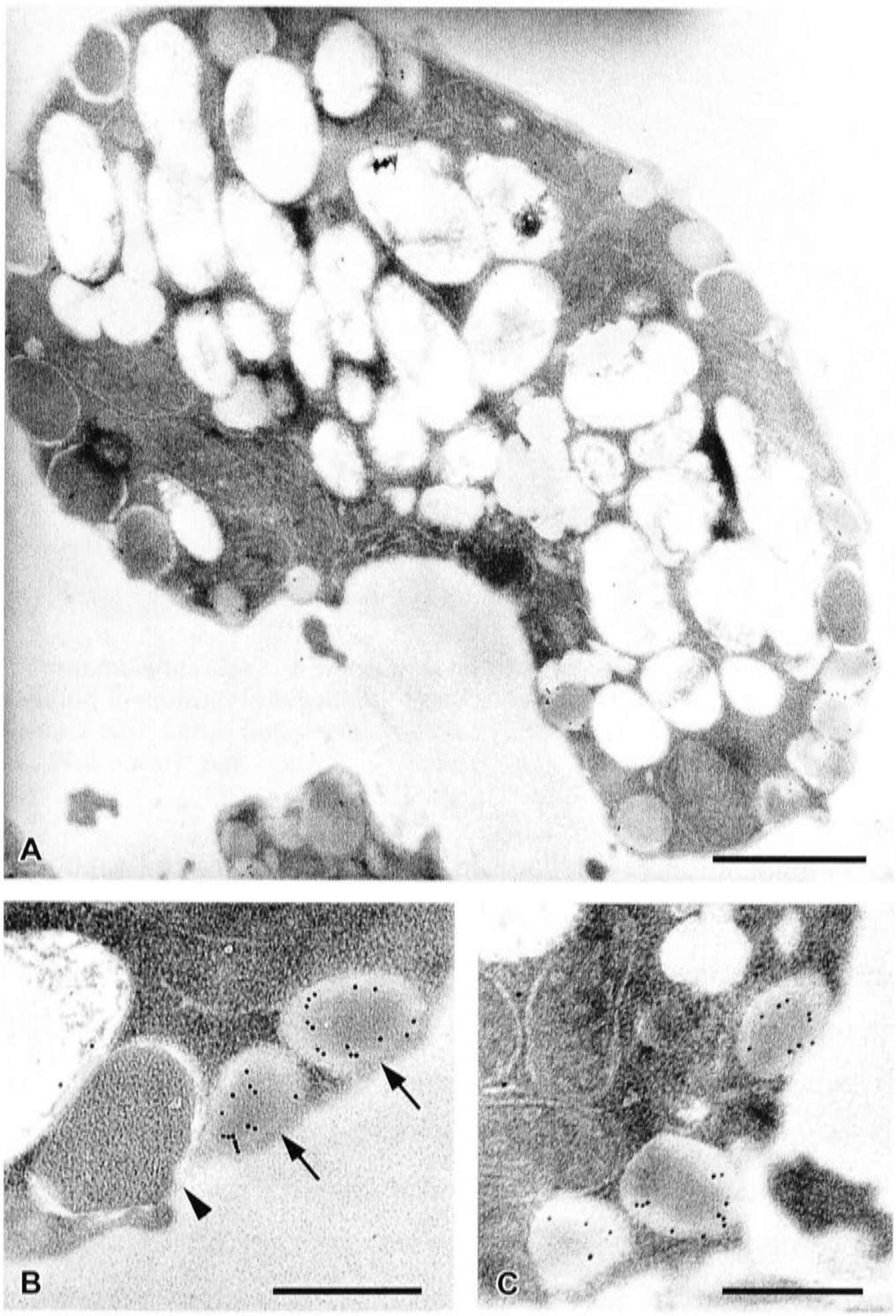

Fig. 4.12 Transmission electron micrograph of *P. nicotianae* zoospores after post-embedding immunogold labelling. Ventral vesicles (arrows) are labelled with Pn-Vsv-4 (A), Pn-Vsv-1 (B), or Pn-Vsv-3 (C). Large peripheral vesicles (arrowhead in B) are not labelled by the Vsv antibodies. Size bars in A: 1 µm, in B and C: 0.5 µm.

The Vsv antibodies were also tested in post-embedding immunogold labelling on sections of *P. cinnamomi* zoospores. Pn Vsv-1, Pn-Vsv-2, Pn-Vsv-3, Pn-Vsv-4, and Pn-Vsv-7 labelled *P. cinnamomi* ventral vesicles in the same way as Vsv-1 (Fig. 4.13). Post-embedding immunogold labelling of *Py. aphanidermatum* resulted in specific labelling of small peripheral vesicles (data not shown).

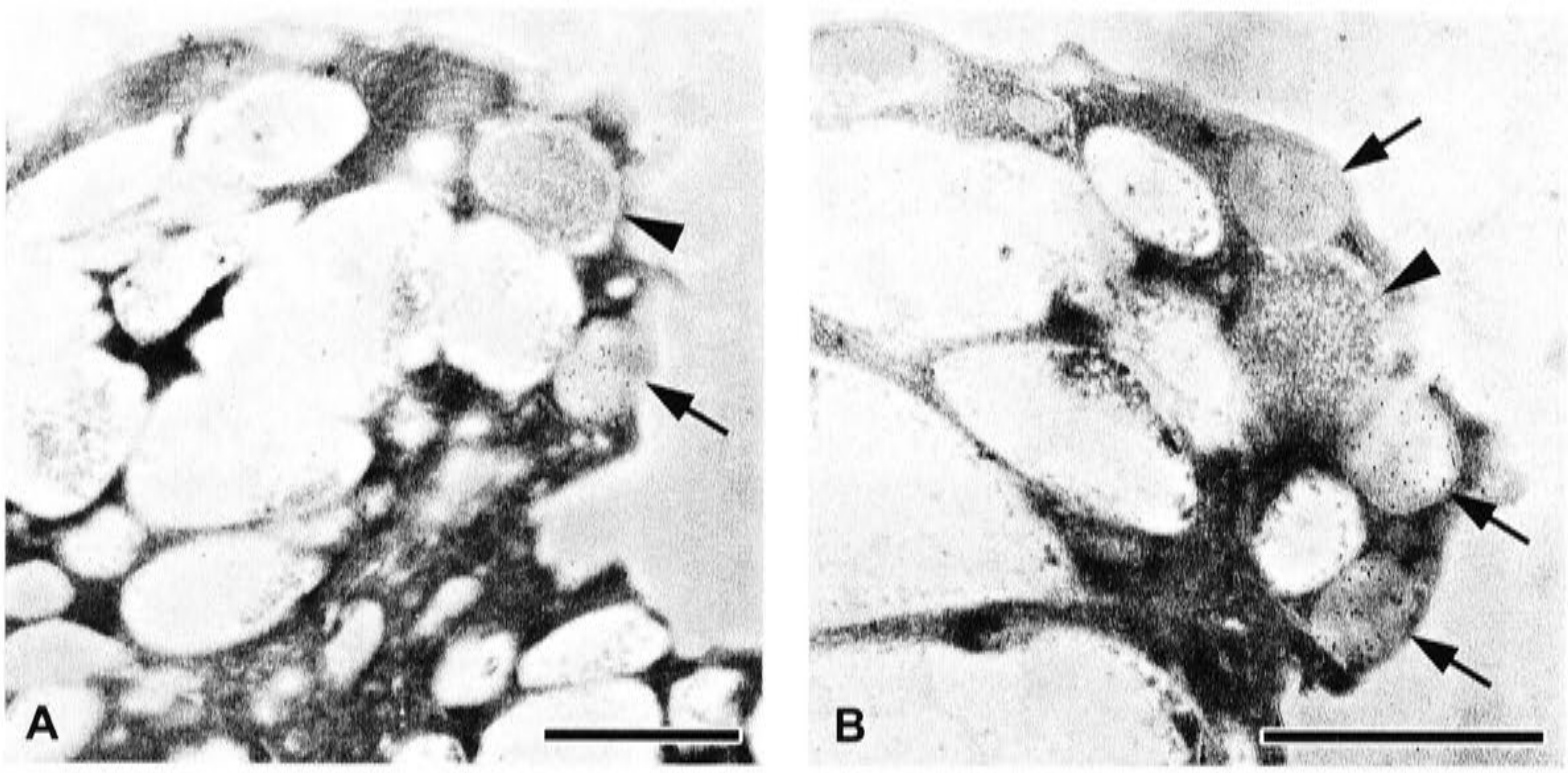

Fig. 4.13 Transmission electron micrographs of *P. cinnamomi* zoospore sections after post-embedding immunogold labelling. Ventral vesicles (arrows) are labelled with Vsv-1 (A) or Pn-Vsv-3 (B). Large peripheral vesicles (arrowheads) are not labelled by the Vsv antibodies. Size bars: 1 µm.

Post-embedding immunogold labelling of sections of *P. nicotianae* zoospores embedded in Lowicryl K4M resin with antibodies Pn-Lpv-1 or Pn-Lpv-2 gave clear labelling of the contents of the large peripheral vesicles in the cell cortex (Fig. 4.14A). In cysts, the labelled vesicles were dispersed throughout the cytosol (Fig. 4.14B). The ultrastructure of formaldehyde fixed zoospores that had been labelled with Pn-Lpv-1 before re-fixing and embedding (pre-embedding labelling) was poorly preserved. However, sections of this material showed gold particles along the outer edge of the large peripheral vesicles (Fig. 4.15). In no case could gold particles be detected inside the vesicles. Post-embedding immunogold labelling of sections of *P. cinnamomi* zoospores with Pn-Lpv-1 or Pn-Lpv-2 lead to labelling of the contents of the large peripheral vesicles (data not shown).

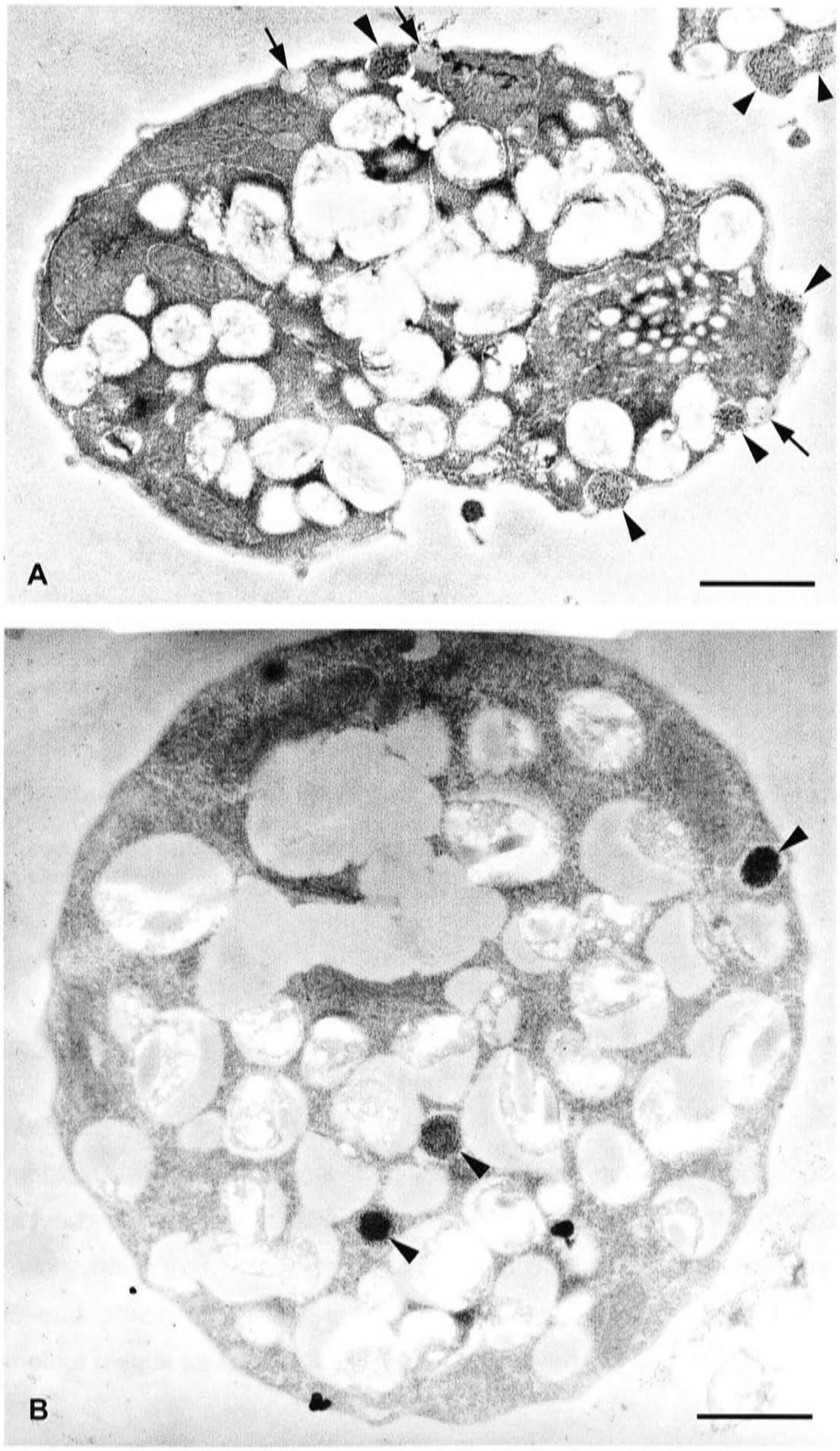

Fig. 4.14 Transmission electron micrographs of *P. nicotianae* zoospore and cyst sections after post-embedding immunogold labelling with Pn-Lpv-1. Large peripheral vesicles (arrowheads) are heavily labelled in sections of zoospores (A) or cysts (B). Ventral vesicles (arrows) are not labelled by the Lpv antibodies. Size bars: 1 µm.

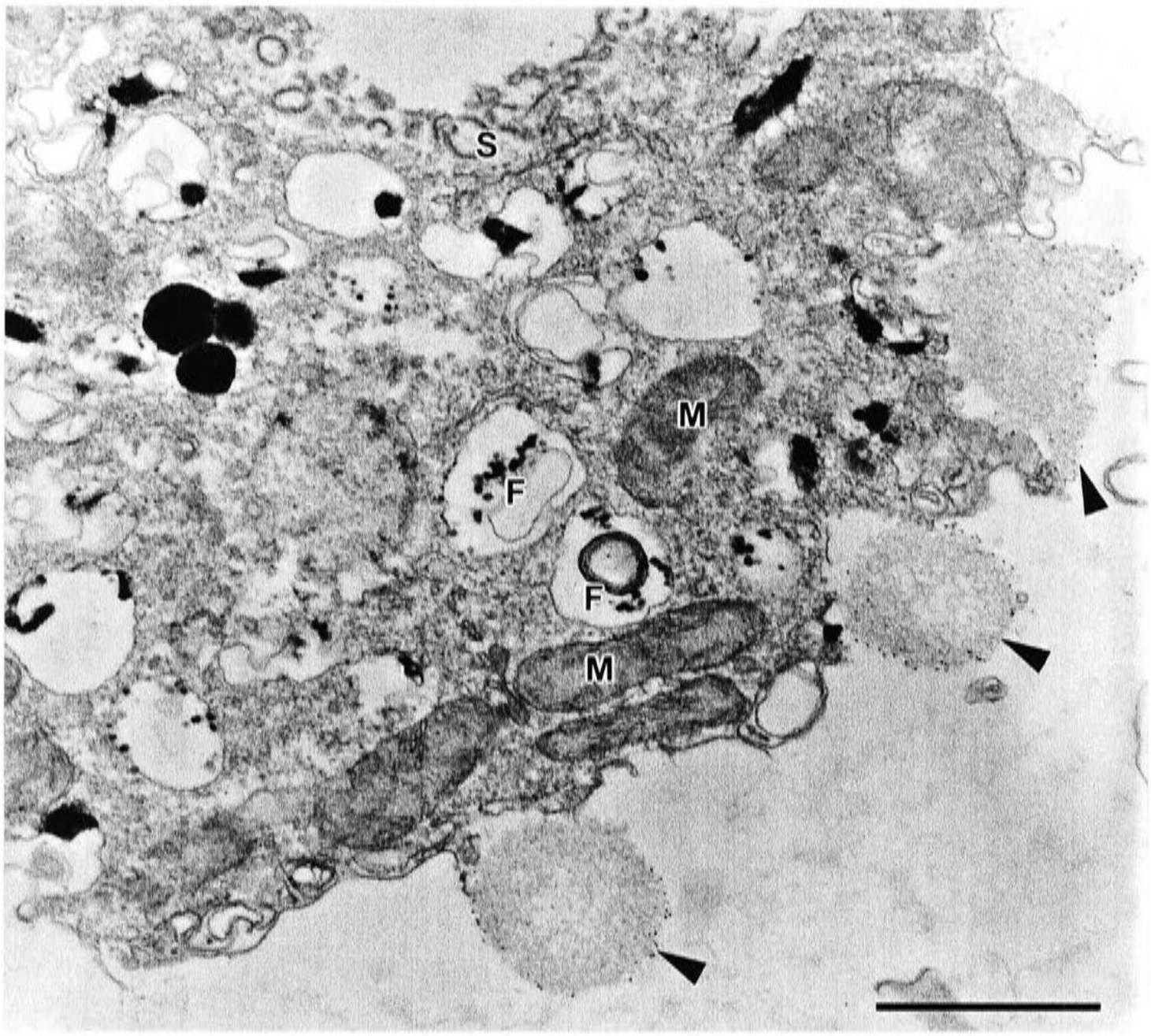

Fig. 4.15 Transmission electron micrograph of part of a *P. nicotianae* zoospore after pre-embedding labelling with Pn-Lpv-1. The cell is poorly preserved; the plasma membrane is fractured. Large peripheral vesicles are bulging out or no longer intact; the outside of the vesicles is labelled with gold particles (arrowheads). Size bar: 1 µm.

4.3.6. Biochemical characterisation of zoospore antigens

Immunoblot

Immunoblots were carried out twice on protein extracts from zoospores, vegetative hyphae, and sporulating hyphae of *P. nicotianae* or from zoospores of *P. cinnamomi* that had been solubilised in 8 M urea, separated by SDS-PAGE and electro-blotted onto a PVDF membrane. A summary of the immunoblot results on *P. nicotianae* material is given in Table 4.2.

Table 4.2: Monoclonal antibody screening results on immunoblots

Monoclonal antibodies labelling the entire zoospore and cyst surface

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
1B4	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
2E5	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
3B3	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
7D8	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
7D9	single band;>200 kDa	negative	single band;>200 kDa
10E7	negative	negative	negative
11B2	single band;>200 kDa	negative	single band;>200 kDa
12E2	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
15G8	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
16D4	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
16D7	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
18E2	negative	negative	negative
18G5	negative	negative	negative
19B10	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands
19C3	wide range; multiple bands	wide range; multiple bands	wide range; multiple bands

Monoclonal antibodies labelling a reticulum within the *P. nicotianae* zoospores

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
9F8	negative	negative	negative

Table 4.2 - continued

Monoclonal antibodies labelling the mastigonemes

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
11G7	negative	negative	negative

Monoclonal antibodies labelling the water expulsion vacuole

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
4D2	negative	negative	negative
5B9	negative	negative	negative
17B3	negative	negative	negative

Monoclonal antibodies labelling the anterior groove

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
3B4	negative	negative	negative
6E9	negative	negative	negative
9C2	negative	negative	negative

Table 4.2 - continued

Monoclonal antibodies labelling few vesicles in the zoospore cortex and the entire cyst surface

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
10F6	double band; approx. 100 kDa	negative	double band; approx. 100 kDa
10F8	negative	negative	negative
12F4	double band; approx. 100 kDa	negative	double band; approx. 100 kDa
13F6	double band; approx. 100 kDa	negative	double band; approx. 100 kDa
14G2	negative	negative	negative

Monoclonal antibodies labelling the ventral vesicles

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
Pn-Vsv-1	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-2	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-3	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-4	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-5	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-6	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-7	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-8	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-9	single band; >200 kDa	negative	single band; >200 kDa
Pn-Vsv-10	negative	negative	negative

Table 4.2 - continued

Monoclonal antibodies labelling the large peripheral vesicles

Antibody	Zoospores/cysts	Vegetative hyphae	Sporulating hyphae
Pn-Lpv-1	two strong bands;>>200 kDa; plus multiple bands; wide ranger	negative	two strong bands;>>200 kDa; plus multiple bands; wide ranger
Pn-Lpv-2	two faint bands; >>200 kDa	negative	negative

Twelve of the 15 monoclonal antibodies directed towards the zoospore and cyst surface were positive on immunoblots of *P. nicotianae* extracts. 10E7, 18E2, and 18G5 were negative, 7D9 and 11B2 labelled a single band with a molecular weight of more than 200 kDa on zoospore extracts, and the remaining 10 antibodies led to strong labelling of multiple bands of various molecular weights (Fig. 4.16). For the latter, the result was the same for zoospores, vegetative hyphae, and sporulating hyphae.

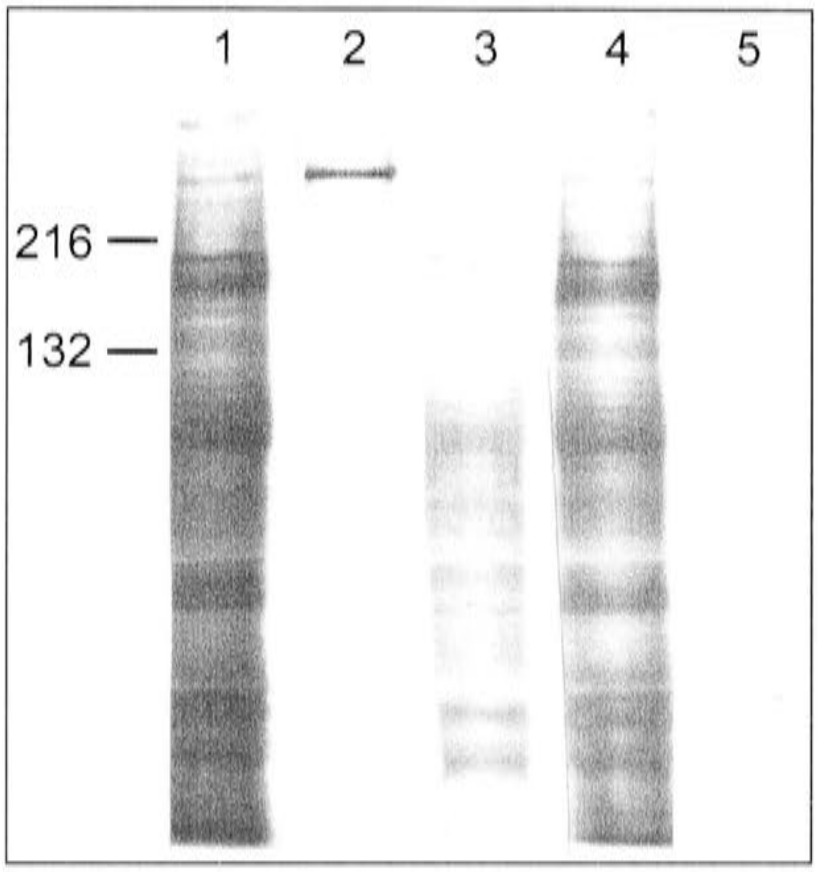

Fig. 4.16 Immunoblots of *P. nicotianae* zoospore proteins separated by 8% SDS-PAGE after incubation with 1B4 (lane 1), 7D9 (lane2), 16D7 (lane 3), 19B10 (lane 4) or without primary antibody (lane 5). Numbers on the left indicate position of molecular weight markers in kDa.

None of the monoclonal antibodies against the reticulum, the mastigonemes, the water expulsion vacuole or the groove led to immunolabelling of immunoblots.

Three of the five antibodies reacting strongly with the entire cyst surface and a few peripheral vesicles in *P. nicotianae* zoospores labelled a double band about 100 kDa in zoospores or sporulating hyphae extracts (Fig. 4.17). The remaining two antibodies, 10F8 and 14G2, were negative in all tested developmental stages.

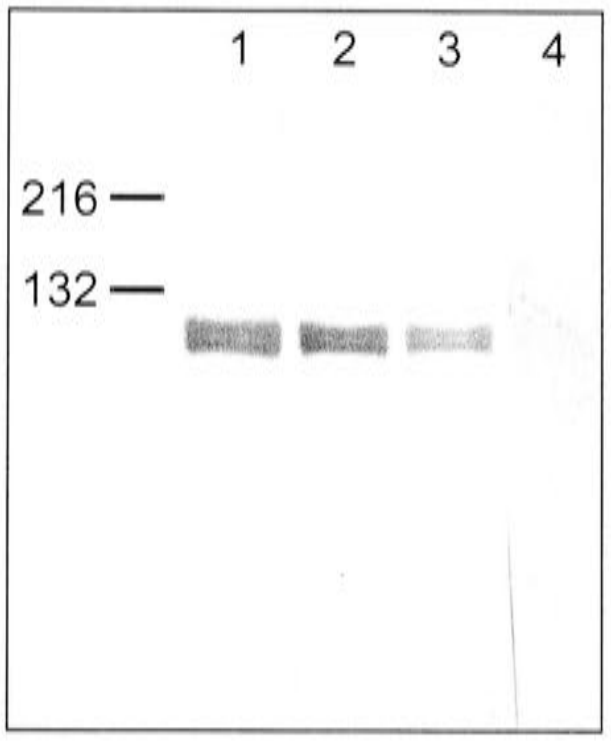

Fig. 4.17 Immunoblots of *P. nicotianae* zoospore proteins separated by 8% SDS-PAGE after incubation with 10F6 (lane 1), 12F4 (lane 2), 13F6 (lane 3) or 14G2 (lane 4). Numbers on the left indicate position of molecular weight markers in kDa.

Of the Vsv antibodies, Pn-Vsv-10 failed to recognise its epitope on immunoblots, whereas the remaining nine Vsv antibodies did react with their antigen(s). The latter labelled a polypeptide with an approx. molecular weight of 230 kDa on immunoblots of *P. nicotianae* zoospore or sporulating hyphae protein extracts (Fig. 4.18). When using *P. cinnamomi* protein extracts Pn-Vsv-1, Pn-Vsv-2, Pn Vsv-3, Pn-Vsv-4, and Pn-Vsv-7 labelled a polypeptide of approx. 220 kDa. A polypeptide of the same molecular weight is recognised by antibody Vsv-1 (Fig. 4.18). All nine monoclonal antibodies failed to recognise a polypeptide on immunoblots of *P. nicotianae* vegetative hyphae. When sporulating hyphae extracts were tested, the nine antibodies recognised their antigen at approx. 230 kDa.

Pn-Lpv-1 and Pn-Lpv-2 reacted with two polypeptides of a relative molecular weight of more than 200 kDa (Fig. 4.19). In the case of Pn-Lpv-1, multiple polypeptides of a wide range of molecular weights were also labelled. Pn-Lpv-2 reacted only with zoospore proteins, Pn-Lpv-1 recognised polypeptides on immunoblots of sporulating hyphae.

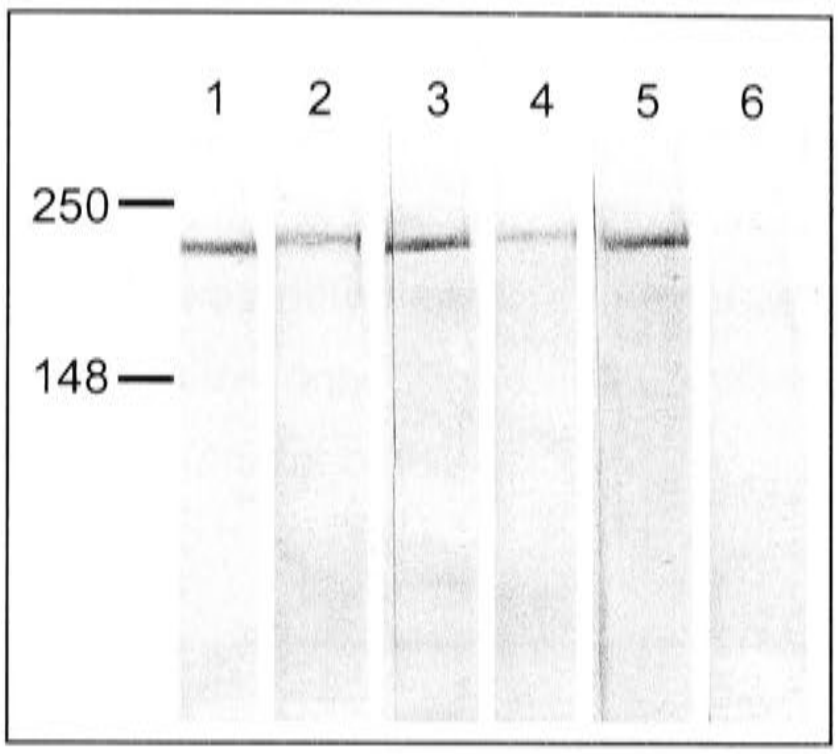

Fig. 4.18 Immunoblots of *P. nicotianae* (lanes 2, 4, and 6) or *P. cinnamomi* (lanes 1, 3, and 5) zoospore proteins separated by SDS-PAGE on a 6% gel after incubation with Pn-Vsv-1 (lanes 1 and 2), Pn-Vsv-2 (lanes 3 and 4) or Vsv-1 (lanes 5 and 6). Pn-Vsv-1 and Pn-Vsv-2 cross-react with their epitope in *P. cinnamomi* while the *P. cinnamomi* antibody Vsv-1 does not recognise its epitope in *P. nicotianae*. Numbers on the left indicate position of molecular weight markers in kDa.

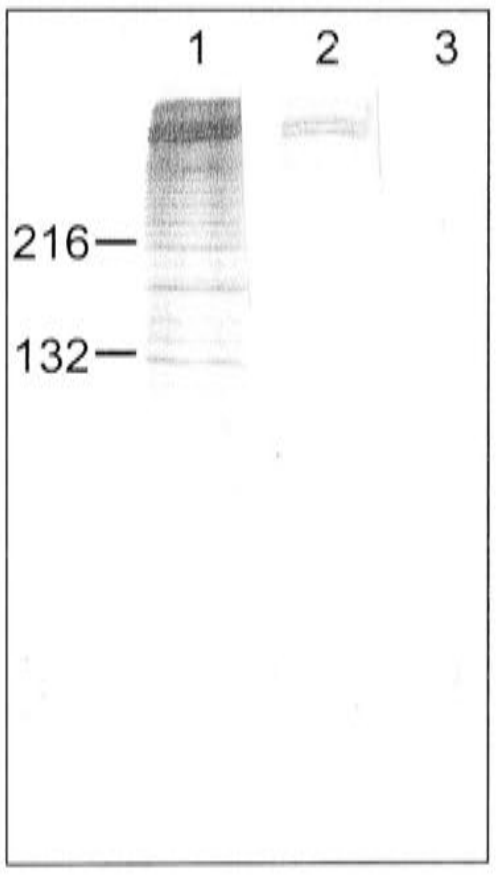

Fig. 4.19 Immunoblots of *Phytophthora nicotianae* zoospore proteins separated by SDS-PAGE on a 8% gel after incubation with Pn-Lpv-1 (lane 1), Pn-Lpv-2 (lane2) or without primary antibody (lane 3). Both antibodies recognise two high molecular weight polypeptides. Numbers on the left indicate position of molecular weight markers in kDa.

Immunodot blot

Immunodot blots were carried out to determine the chemical nature of the epitopes of the Vsv antibodies. Monoclonal antibodies Pn-Vsv-1 to Pn-Vsv-9 recognised an epitope that was only sensitive to treatment with pronase, thus the epitope consisted of protein only (Fig. 4.20). Antibody Pn-Vsv-10 did not recognise its epitope on immunodot blots.

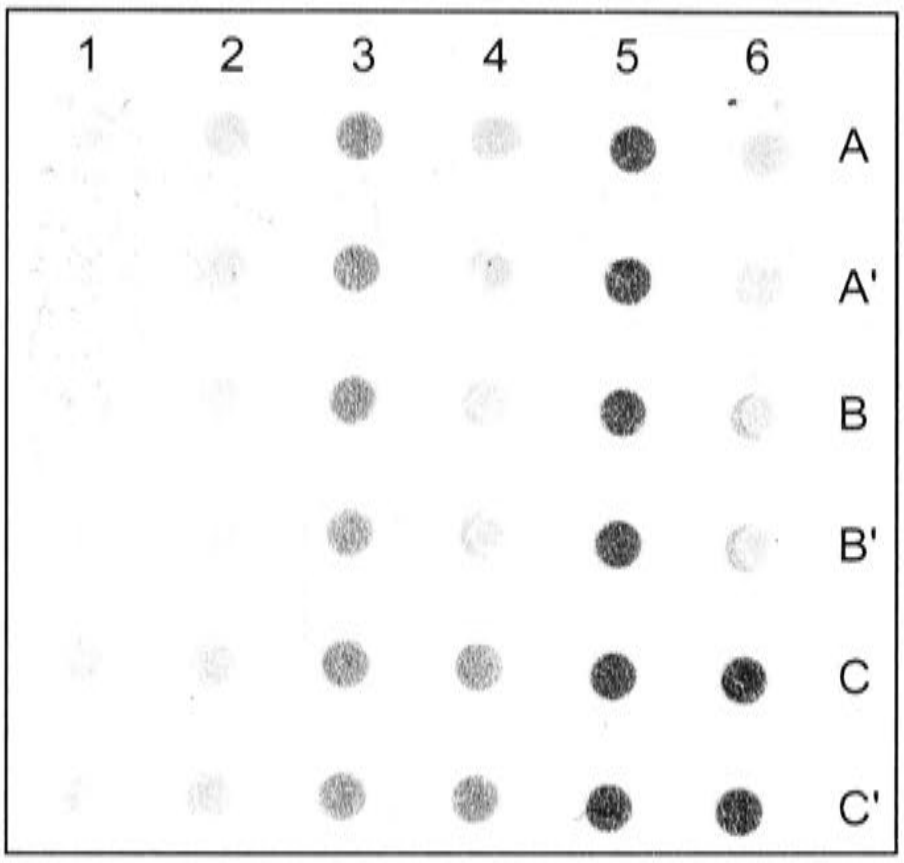

Fig. 4.20 Immunodot blot showing antibody labelling after pronase or periodate treatment of *P. nicotianae* zoospore protein extracts. Columns 1 and 2 show results after incubation without primary antibody, columns 3 and 4 after labelling with Pn-Vsv-2, and columns 5 and 6 after labelling with Pn-Vsv-3. Columns 1, 3, and 5 have been treated with periodate or acetate buffer, columns 2, 4, and 6 with pronase or PBS. Rows A and A' are duplicates treated with either 20 mM periodate (1, 3, and 5) or 1 mg pronase/mL (2, 4, and 6). Rows B and B' are duplicates treated with 10 mM periodate (1, 3, and 5) or 100 µg pronase/mL (2, 4, and 6). Rows C and C' are duplicates treated with acetate buffer (1, 3, and 5) or PBS (2, 4, and 6). Antibody labelling is abolished by pronase digestion of the antigen(s) (columns 4 and 6).

4.4 Discussion

4.4.1. Monoclonal antibodies directed towards *Phytophthora nicotianae* zoospores were generated using a co-immunisation protocol

Zoospores are the main infective stage of most oomycete species during disease favouring conditions. For this reason, the characterisation of zoospore components can significantly increase our understanding of disease development. In the current study, a co-immunisation procedure was employed to generate monoclonal antibodies specific for *Phytophthora* components that occur only during sporulation. These antibodies can be used in a variety of assays to characterise their antigen, including microscopic, biochemical, and molecular biological studies. In the initial screen which used indirect immunofluorescent assays, 90 out of 364 hybridoma cell lines (25%) reacted positively with *P. nicotianae* zoospores and/or cysts. This percentage of positive clones was high when compared to other fusions carried out in our laboratory. The second most successful fusion with respect to the number of monoclonal antibodies obtained was fusion II described in Hardham et al. (1991b) with 50 positives out of 383 cell lines (13%). In addition, the antibodies obtained in the present study displayed a wide variety of labelling patterns in immunofluorescence assays. The immunofluorescence labelling patterns indicate that at least eight different antigens were recognised by the 40 antibodies that were kept and further characterised (Table 4.1). Only the fusion reported in Hardham et al. (1986) yielded monoclonal antibodies giving the same diversity of immunofluorescence patters. The success of the current fusion could be due to the accessibility of antigens in the microsomal preparations used in the immunisations. The use of microsomal preparations in the immunisations could also explain the finding of monoclonal antibodies against both surface and intracellular antigens.

4.4.2. The monoclonal antibodies generated in the current study recognise a range of antigens

Of the 40 antibodies characterised in indirect immunofluorescence assays, 28 (70%) exclusively showed labelling of developmental stages involved in asexual spore production, namely zoospores, cysts, and/or sporulating hyphae. The high percentage of antibodies specific for sporulation is probably due to the co-immunisation procedure, a technique that has already yielded useful monoclonal antibodies in a number of studies of oomycete and fungal plant pathogens. In one study which aimed at the generation of antibodies that were specific for the species *P. nicotianae* five out of nine (56%) antibodies isolated were species-specific (Robold and Hardham 1998). The approach followed was to inject the first set of mice with *P. cinnamomi* and *P. cryptogea* cysts. A second set of mice was inoculated with a *P. nicotianae* cyst preparation mixed with serum from the first mice. An increase in the number of monoclonal antibodies specific for a certain developmental stage using co-immunisation was also recorded by Pain et al. (1994) where, although the cell type of interest constituted only approx. 3% of the antigen, 18% of the monoclonal antibodies were specific for this cell type.

4.4.3. Monoclonal antibodies directed towards components present in the surface of zoospores and cysts

In the present study, one group of monoclonal antibodies labelled the entire surface of zoospores and cysts in indirect immunofluorescence assays, a pattern comparable to that obtained with the ZCp antibodies raised against *P. cinnamomi* (Hardham et al. 1986 and 1991b) and antibodies PA7 and PA8 raised against *Py. aphanidermatum* (Estrada-Garcia et al. 1989). The antibodies generated in the current study are referred to as Pn-ZCp antibodies. In *P. nicotianae*, the Pn-ZCp antigen dominates the immune response in injected animals. In the fusions reported in Gautam et al. (1999), 10 out of 41 monoclonal antibodies (25%) were directed towards antigens present on the surface of *P. nicotianae* zoospores and cysts. In the present study, 15 out of 40 monoclonal antibodies showed this labelling pattern (37.5%; Fig. 4.3). Mitchell

et al. (2002) found that antibodies raised towards components on the surface of zoospores and cysts of *P. nicotianae* by Gautam et al. (1999) can be further sub-divided according to their labelling pattern on immunoblots and in indirect immunofluorescence and immunogold assays. One third of the Pn-ZCp antibodies produced in the present study (five antibodies) are specific for asexual sporulation. The antigen is present in sporulating hyphae, zoospores, and cysts but absent in vegetative hyphae. The 10 other antibodies in this group also reacted with components in vegetative hyphae and are thus not specific for sporulation. Nine of these 10 gave rise to high background fluorescence, as did the ZCp monoclonal antibodies obtained by Hardham et al. (1986). All monoclonal antibodies that were not specific for sporulation recognised a large number of polypeptides on immunoblots, a feature similar to that shown by Group 3 antibody, Pn6G9, in the study of Mitchell et al. (2002). Two of the five sporulation-specific monoclonal antibodies (7D9 and 11B2) generated in the current study were positive when tested on immunoblots. They reacted with a polypeptide larger than 200 kDa. Their labelling pattern on immunoblots and in immunofluorescence assays was identical to that of monoclonal antibodies Pn3B3 and Pn3C3 described by Mitchell et al. (2002).

4.4.4. A monoclonal antibody directed towards a component of a reticulum present in zoospores

One monoclonal antibody, 9F8, produced in the present study, recognised a reticulum in zoospores. Five monoclonal antibodies directed towards a reticulum in *P. cinnamomi* were isolated in fusions specifically aimed at generating probes towards intracellular antigens (Hardham et al. 1991b). It is possible that the component being labelled is present in the endoplasmic reticulum. Testing of 9F8 in indirect immunofluorescence assays with different developmental stages indicated that it is specific for asexual sporulation. Unfortunately, the antibody failed to recognise its epitope on immunoblots. This fact is disappointing as information on the endoplasmic reticulum and Golgi apparatus – despite their importance – is still limited in *Phytophthora*. The endoplasmic reticulum and Golgi apparatus have been shown to synthesise

Lpv, Vsv, and Cpa antigen concurrently in *P. cinnamomi* (Dearnaley and Hardham 1994).

4.4.5. A monoclonal antibody against a mastigoneme component

A single monoclonal antibody, 11G7, directed towards the mastigonemes present on the anterior flagellum of zoospores was obtained. Flagella and mastigonemes constitute only a small portion in the antigen mixture when whole or ground zoospores are used for immunisation. At the same time, flagella and their appendages are generally well preserved during routine fixations that include glutaraldehyde. In addition, their location on the spore surface makes them easily accessible to the immune system of the injected animal. Taken together, one would expect a low number of antibodies towards flagellar antigens in most fusions where whole fixed zoospores and cysts are used in the immunisations. Indeed, a low number of monoclonal antibodies directed to flagellar components was isolated in most studies in which the antigenic preparation contained *Phytophthora* or *Pythium* zoospores (e.g. *P. cinnamomi*, Hardham et al. 1986; *Py. aphanidermatum*, Estrada-Garcia et al. 1989). However, the expectation to obtain a low number of monoclonal antibodies directed towards flagellar antigens is in contrast to the first of three fusions against 90% zoospores and 10% cysts reported in Gautam et al. (1999). In the study by Gautam et al. (1999), an unusually high proportion of antibodies were directed towards mastigoneme antigens (over 50% of the positive hybridoma supernatants). However, antibody 11G7 produced in the current study was negative on immunoblots. So far, only one monoclonal antibody, Pn14B7, has been produced that recognises its antigen on immunoblots (Robold and Hardham 1998); all other antibodies against flagellar components in *P. cinnamomi* or *P. nicotianae* have failed to react on immunoblots. Either the steric properties of most antigenic epitopes are destroyed as the proteins are linearised in the preparation for SDS-PAGE or the amount of antigen on immunoblots leads to a signal that is below the detection level. The former problem could be overcome by employing native gels, the latter by loading more

protein extract per lane, by isolating and loading flagella only (Gubler et al. 1990) or by 2D-gel electrophoresis.

4.4.6. Monoclonal antibodies directed towards components present in the water expulsion vacuole

Three monoclonal antibodies (4D2, 5B9, and 17B3) recognised the water expulsion vacuole in either mature zoospores within sporangia or in free-swimming zoospores. Only one antibody recognised its epitope in sporulating hyphae. None of the monoclonal antibodies reacted with vegetative hyphae. The water expulsion vacuole is a highly specialised structure (Mitchell and Hardham 1999) and it should therefore be possible to generate a number of monoclonal antibodies directed towards the different components contributing to this specialisation. However, only two antibodies against the water expulsion vacuole in *P. cinnamomi* (Hardham et al. 1991b) and another two in *P. nicotianae* (Gautam et al. 1999) have been reported to date. None of these antibodies react on immunoblots, so the biochemical nature of their epitopes is still elusive (Mitchell et al. 2002; Prof. A.R. Hardham, personal communication).

4.4.7. Monoclonal antibodies against antigens present in the ventral groove of zoospores

The labelling pattern obtained in indirect immunofluorescence assays with monoclonal antibodies 3B4, 6E9, and 9C2 is reminiscent of the labelling pattern obtained with antibody Zg-1 in the anterior part of the ventral groove of *P. cinnamomi* zoospores (Hardham et al. 1991b). However, *P. cinnamomi* antibody Zg-1 did not recognise the posterior part of the groove that is also labelled by antibodies 3B4, 6E9, and 9C2 in many cases (Prof. A.R. Hardham, personal communication). The antigens of the first two antibodies were also detectable in sporulating hyphae. All three are sporulation-specific antibodies. The antibodies were negative on immunoblots, so the biochemical nature of their antigen(s) is unknown.

4.4.8. Monoclonal antibodies that potentially recognise a Cpa homologue in *Phytophthora nicotianae*

Five monoclonal antibodies (12.5%) reacted strongly with material present on the cyst surface and also in a few vesicles in the zoospore periphery. In *P. cinnamomi*, this labelling pattern has so far not been reported. Monoclonal antibodies labelling the cyst surface in *P. cinnamomi* have been divided into three groups (Hardham et al. 1991b). (I) ZCp antibodies have been described above; they label the surface of zoospores in addition to the cyst surface. (II) Cpw antibodies exclusively label the cyst surface, and (III) Cpa antibodies react with the small dorsal vesicles of zoospores and – after release of the contents of the dorsal vesicles during encystment – the cyst surface. The labelling pattern of the antibodies produced in the current study on zoospores and cysts of *P. nicotianae* is most similar to the one described for the Cpa antibodies in *P. cinnamomi*. However, the number of strongly labelled vesicles in *P. nicotianae* zoospores is much lower than in *P. cinnamomi*. This observation is consistent with the lower number of dorsal vesicle profiles in ultrathin sections of *P. nicotianae* zoospores when compared to *P. cinnamomi* indicating that the antibodies might react with a *P. nicotianae* Cpa equivalent. In addition, the vesicular membrane of the dorsal vesicles in *P. nicotianae* might be better preserved than in *P. cinnamomi* by routine formaldehyde fixation. This could explain the fact that only a few vesicles are strongly labelled whereas most show only weak fluorescence. Unfortunately, the antibodies failed to cross-react with the antigen present in *P. cinnamomi* zoospores, so direct comparison is not possible (Prof. A.R. Hardham, personal communication). However, the labelling pattern of the antibodies in immunofluorescence assays is similar that obtained with antibodies PA3 to PA6 on *Py. aphanidermatum* cells (Estrada-Garcia et al. 1989). The authors concluded from their observations that in *Py. aphanidermatum* the antigenic material is released onto the cyst surface during encystment. As mentioned above this is also true for the Cpa antigen that is stored in the dorsal vesicles in *P. cinnamomi* zoospores. In *P. cinnamomi* and *Py. aphanidermatum* this antigen dominates the immune response in conventional immunisations and also after neonatal tolerisation with cysts (Estrada-Garcia et al. 1989; Hardham et al. 1991b), but until the present study

in *P. nicotianae* not a single antibody towards the dorsal vesicles has been obtained. Indirect immunogold labelling of *P. nicotianae* zoospore sections with the five new antibodies failed, so the ultrastructural localisation of the antigen is still unknown. Three of the antibodies reacted with two polypeptides of approx. 100 kDa in *P. nicotianae* zoospore or sporulating hyphae protein extracts. In *P. cinnamomi*, monoclonal antibody Cpa-2 labels three high molecular weight bands above 200 kDa (Gubler and Hardham 1988). In *Py. aphanidermatum* and *Py. butleri* it recognises two polypeptides larger than 200 kDa (Cope et al. 1996). It is possible that the dorsal vesicles of different oomycete species either contain a panel of different proteins and only a subset is highly immunogenic or they contain different antigens in the different species. The three monoclonal antibodies produced in the current study that are positive on immunoblots should be tested on pronase or periodate treated protein samples to gain evidence about the nature of their epitopes. If they reacted with a protein epitope, they could be used to obtain information about the genes encoding their antigens.

4.4.9. Monoclonal antibodies against components present in the large peripheral vesicles

Two monoclonal antibodies (5%) against the large peripheral vesicles in *P. nicotianae* and *P. cinnamomi* were generated. In post-embedding immunogold labelling with Pn-Lpv-1, clear labelling of large peripheral vesicles in *P. cinnamomi* and *P. nicotianae* zoospores and *P. nicotianae* cysts was observed. In pre-embedding labelling of *P. nicotianae* zoospores the large peripheral vesicles were recognised. The fact that pre-embedding immunogold experiments resulted in labelling of the outermost area of the large peripheral vesicles might be due to the accessibility of the antigen to the antibody. Pn-Lpv-1 is an IgM and the mature immunoglobulin has a molecular weight of approx. 1500 kDa (Harlow and Lane 1999); it constitutes a large molecule and its size might hinder it from accessing the antigen within the vesicles during pre-embedding labelling. Also, if the large peripheral vesicles - as proposed by Gubler and Hardham (1988 and 1990) and Marshall et al. (2001) - were storage

vesicles, one would expect their contents to be tightly packed and the antibody would be unable to access its antigen in the inner part of the vesicles. Both monoclonal antibodies were positive on immunoblots, Pn-Lpv-1 reacted very strongly. The antibodies labelled two high molecular weight polypeptides in *P. nicotianae* zoospores. In extracts of *P. cinnamomi*, Lpv-1 recognises three high molecular weight bands (Gubler and Hardham 1988). The fact that two bands are recognised in *P. nicotianae* and three bands in *P. cinnamomi* zoospores might be due to the presence of only two instead of three types of polypeptides in *P. nicotianae* large peripheral vesicles. Another explanation could be that the epitope recognised by Pn-Lpv-1 and Pn-Lpv-2 is only present in two out of three (or more) types of polypeptides in the large peripheral vesicles.

4.4.10. Monoclonal antibodies directed towards an antigen present in the ventral vesicles

Ten monoclonal antibodies (25%) were directed towards an antigen present in the ventral vesicles of *P. nicotianae* zoospores and sporulating hyphae. Nine of these cross-reacted with the contents of the ventral vesicles in *P. cinnamomi* zoospores. Before this study, only two monoclonal antibodies against the ventral vesicles of *P. cinnamomi* zoospores had been generated and this was following an immunisation protocol employing neonatal tolerisation with *P. cinnamomi* cysts (fusion III, Hardham et al. 1991b). The *P. nicotianae* Vsv antibodies did not react with vegetative hyphae of *P. nicotianae* indicating that the recognised antigen is produced during sporulation as it has been demonstrated for the Vsv-1 antigen in *P. cinnamomi* (Dearnaley et al. 1996). Several of the antibodies recognised their epitope in post-embedding immunogold labelling and in this way the ultrastructural localisation of the antigen within the ventral vesicles in *P. nicotianae*, *P. cinnamomi*, and *Py. aphanidermatum* could be confirmed. All but one of the antibodies were positive on immunoblots of *P. nicotianae* zoospores and sporulating hyphae, labelling a polypeptide with a molecular weight larger than 200 kDa. Several of these cross-reacted with the antigen in *P. cinnamomi* zoospores that is also recognised by antibody Vsv-1 (Hardham and Gubler 1990). From this result it is

concluded that the Vsv antibodies produced in this study recognise at least three different epitopes within the antigen: one of these is present in both species and not recognised on immunoblots, one is present in both species but only recognised on immunoblots of *P. nicotianae* protein extracts, and one is present in *P. nicotianae* and *P. cinnamomi* zoospores and positive on immunoblots of protein extracts from both species.

The co-immunisation procedure utilised in the present study has yielded a number of monoclonal antibodies that target interesting antigens. It would be well worth investigating the nature of the antigens further and attempting to optimise the experimental procedures for immunoblots after this initial screen for each antigen that could not be detected by their antibody. For further analysis, the group of antibodies recognising an antigen in the ventral vesicles was chosen. The Vsv protein is thought to be important for the adhesion of *Phytophthora* cysts to their potential host (Hardham and Gubler 1990). Adhesion is likely to play a crucial role in the early infection process of many pathogens to their hosts (see following chapter), so my work focussed on the Vsv antibodies and their antigen. Testing of the antibodies revealed that the epitopes consisted of protein, and the antibodies could be used for immunological screening of expression libraries of the appropriate developmental stage, sporulating hyphae. Nevertheless, it would be of great value if other antibodies produced in the present study could be used to characterise their antigens in more detail.

Chapter 5 Cloning and sequencing of a putative adhesive in *Phytophthora cinnamomi*

5.1 Introduction

Adhesion is a fundamental requirement in both single and multicellular organisms. Higher organisms (plants and animals) have a wide variety of adhesive molecules that are often also involved in guidance of cells and tissue or organ development (some examples are reviewed by Wedlich 2002 or Boekel and Brown 2002). Recent interest has developed in the evolution of molecules responsible for attachment (e.g. reviewed by Hynes and Zhao 2000, Mueller et al. 2001 or Hutter et al. 2000). In animal cells a multitude of adhesion molecules is known. Many of the adhesives can be grouped into protein or glycoprotein superfamilies. Often members of the same family have different properties that make them suitable for their particular function. Adhesive components are involved in complex and essential processes, for example, in neural crest cell migration and neurite guidance. Molecules mediating cell-cell adhesion and molecules mediating cell-matrix adhesion are known.

Many extracellular matrix (ECM) proteins have a function in cell adhesion, but their importance in cell growth and morphology as well as cell development has also been recognised. Often these molecules are large and made up of several subunits. Prominent members are, for example, fibronectins, laminins, tenascin, thrombospondins, and vitronectins. The glycoproteins termed fibronectins in addition to their cell adhesive function also affect cell morphology, cell migration, differentiation, and cytoskeletal organisation (reviewed by Hynes 1993). Laminins are made of an A and two B subunits. Apart from cell adhesion, they are involved in cell migration, growth, and differentiation (reviewed by Engval 1993). Tenascin is a hexameric ECM glycoprotein and affects via cell adhesion, cell morphology, and cell differentiation (reviewed by Chiquet-Ehrismann 1993). Thrombospondins are

glycoproteins with a modular make-up (Adams 2001). For example, thrombospondin-1 is a 420 kDa glycoprotein composed of three equal molecular weight subunits and is expressed at high levels in developing heart, muscle, brain, and bone (reviewed by Lawler 1993). It is composed of several different domains that make binding to a wide variety of molecules such as syndecan, heparan sulfate, integrins, decorin, fibronectin, and laminin possible (reviewed by Adams and Tucker 2000). Vitronectin is a protein that strongly adheres to cells and is abundant in blood plasma (reviewed by Mosher 1993). The importance of adhesive molecules in animals including humans is demonstrated dramatically by a 400 to 10000 kDa multimeric glycoprotein that mediates platelet adhesion and thrombus formation, the von Willebrand Factor. The molecule plays an important role in the arrest of bleeding, for example, due to vascular injury. The inheritable von Willebrand disease is caused by abnormalities in this blood component (reviewed by Ruggeri and Ware 1993).

Most molecules that mediate cell adhesion and cell-to-cell contact belong to the immunoglobulin, cadherin, selectin, and H-CAM superfamilies (reviewed by Oebrink 1993). Most of them have a membrane-spanning domain that makes interaction with ECM and intracellular components, for example, the cytoskeleton possible. Proteins of the immunoglobulin superfamily are characterised by an immunoglobulin domain of about 100 amino acid residues. They are important in embryonic development but also in adult tissue, including cells of the nervous system, various epithelia, and leukocytes. Some of these proteins are calcium-independent, for example, N-CAM, and hence important adhesives of the calcium-independent pathways. Members of the cadherin superfamily are all strictly calcium-dependent, single-pass transmembrane proteins (reviewed by Alberts et al. 2002). Some of them are directly involved in cell-cell adhesion. With their cytoplasmic domain, they can interact with microfilaments or intermediate filaments. An essential role in tissue formation during embryonic development has been shown, but also adult tissues including neural, muscular, and epithelial tissues have cadherins. Integrins are heterodimers consisting of one α- and one β-chain which both are single-pass transmembrane proteins. Some integrins are known to interact with cytoskeletal elements. A wide variety of integrins can be made due to various combinatory possibilities of the different α- and β-chains. Members of the

selectin superfamily are present in leukocytes, platelets, and vessel endothelial cells. These single-pass transmembrane proteins have a lectin-like domain in their extracellular portion that has been proved to bind specific carbohydrate ligands. Proteins of the H-CAM superfamily again are single-pass transmembrane proteins. Their sequences are characterised by extracellular motifs that are homologous to the proteoglycan core protein and the link protein of cartilage.

In the case of plants, recent advances are starting to unravel the molecules and mechanism involved in adhesion. In higher plants adhesion between cell walls, between cell walls and plasma membrane, and during pollination has received attention. The adhesion mechanisms of cells of higher plants differ markedly from those in animal cells: plant cells are surrounded by cell walls and these adhere to each other along the middle lamella. Cell division can be followed by regulated cell separation as the cell develops within the forming tissue. Once a cell is detached from the parent cell it cannot aggregate, another feature that differs fundamentally from animal cells. The major constituents of the middle lamella as well as the primary cell wall and the cell corners are pectic polysaccharides (for reviews see Knox 1992 and Williats et al. 2001). Pectin synthesis is a complex process requiring at least 53 different enzymes (reviewed by Ridley et al. 2001). Iwai et al. (2002) identified a gene encoding a pectin glucuronyltransferase in *Nicotiana plumbaginifolia* using T-DNA transformation and subsequent *in-vitro* culture of the transformants. The glucuronyltransferase-deficient mutant formed calli with loosely attached cells and severely impaired growth when haploid plants were regenerated indicating a fundamental role of pectin in the intercellular adhesion of plants. The adhesion of plant cell walls to the plasma membrane is another exciting field of current investigations. Upon plasmolysis Hechtian strands are visible that connect the plasma membrane to the cell wall. On onion bulb epidermal cells these connections disappear after incubation with RGD-containing peptides (Canut et al. 1998). Zhu et al. (1993) found that isolated protoplasts of tobacco cells adapted to high concentrations of NaCl adhered tightly to each other whereas protoplasts of non-adapted cells failed to adhere. Addition of an RGD-containing peptide blocked adhesion implicating the presence of an integrin-related system in plasma membrane attachment. Laval et al. (1999) found

genes in *Arabidopsis thaliana* that encode proteins with a limited similarity to animal integrins. These integrin-like molecules seem to be involved in physiological plant responses like graviperception and pathogen-related defence (Wayne et al. 1992, Mellersh and Heath 2001). The adhesion of pollen to the stigma and of pollen tubes during their growth towards the ovule has been addressed in recent research. Using an *in-vitro* assay it has been demonstrated that the rate of lily pollen growth is higher than in conventional *in-vitro* assays when the tip of the pollen tube can adhere to stylar exudate (Jauh et al. 1997). Subsequently, a stigma/stylar cysteine-rich adhesin (SCA) was isolated that causes pollen adhesion *in-vitro*; *in-vivo* the SCA in combination with a pectic polysaccharide seems to guide the pollen tube towards the ovule, so an additional role of adhesives in plant development seems clear (Park et al. 2000, Mollet et al. 2000, reviewed by Lord 2003). Molecules involved in pollen/stigma adhesion are currently being characterised in *Brassica spp*. and *Arabidopsis thaliana* (Luu et al. 1999, Zinkl et al. 1999).

Chlamydomonas is a unicellular green alga and investigation of its life cycle has revealed that adhesion is of fundamental importance during fertilisation (Pan and Snell 2000). Gametes of both mating types display agglutinins on their flagellar surfaces. These high molecular weight glycoproteins are known as mt+ and mt- agglutinins depending on the mating type. In the first step of mating the gametes stop swimming and their flagella adhere probably due to interaction of their agglutinins. Flagellar attachment stimulates the gametes and after a dramatic increase in cyclic adenosine mono-phosphate concentration the adhesiveness of the flagella increases eight to ten-fold (Goodenough 1989; Demets et al. 1988). This strong increase in adhesion seems to be due to the translocation of inactive agglutinin molecules from the cell body onto the flagellar surface where the agglutinins become active (Snell and Moore 1980). The two gametes of the opposite mating type bring their cell apices closely together and mt+ and mt- gametes produce fertilisation tubes of different length and structure. The gametes adhere on the tips of these tubes with active adhesion/fusion molecules that differ from the flagellar agglutinin molecules. Using fusion-deficient mutants, the candidate adhesion/fusion gene FUS1 has been cloned in *C. reinhardtii* (Ferris et al. 1996). The gametes eventually fuse to form a zygote (reviewed in Pan and Snell 2000).

The bio-fouling green alga *Enteromorpha* produces quadriflagellate zoospores that adhere to substrata, retract their flagella, form a cell wall, and eventually develop a new thallus. *In-vitro* it favours hydrophobic surfaces for attachment (Callow et al. 2000a). Atomic force microscopy studies have demonstrated the formation of an adhesive pad between zoospore and substratum upon adhesion (Callow et al. 2000b). Monoclonal antibodies Ent1 and Ent6 have been generated; they react with a 110 kDa glycoprotein that is secreted upon settlement of the zoospore. The antigen is also present in the newly formed cell wall and could represent the adhesive as both monoclonal antibodies specifically decrease adhesion (Stanley et al. 1999). The same study showed that the apparent molecular weight of the glycoprotein decreases to approx. 95 kDa upon Peptide N-glycosidase treatment indicating the presence of N-linked sugars. In studies with concentrations of 10µg mL^{-1} of the Golgi apparatus-affecting drug Brefeldin A adhesion is reduced approx. 46% after 2h, suggesting the involvement of the glycosyslation and secretory pathway in the generation of the adhesive (Callow et al. 2001).

Apart from a role in cell morphology, development, and mating, adhesives are very important in pathogenic organisms. In pathogens, secretion of adhesive molecules often precedes and facilitates successful infection. Therefore, a brief overview on adhesives in microscopic organisms shall be given in the next few paragraphs.

5.1.1. Adhesion in human pathogenic bacteria

Human pathogenic bacteria have been extensively researched. For example, uropathogenic strains of *E. coli* enter the bladder via the urethra and adhesive molecules, termed adhesins, are thought to be the most important pathogenicity determining factors for successful infections (reviewed by Mulvey 2002). The direct effect of the adhesins lies in anchoring the bacteria to the host cells, thereby preventing the removal of the pathogen with the bulk flow of urine. However, it should be mentioned that adhesins are also indirectly important for successful colonisation, e.g. by triggering host and pathogen cell signalling pathways or facilitating the delivery of other bacterial products to the host cells.

Adhesion molecules of the Dr adhesin family of uropathogenic strains of *E. coli* contribute significantly to the persistence of the pathogen in infected kidneys. In these cases, the pathogen can persist for more than one year in renal tissue. Other adhesins form composite fibres, for example, type-1 pili, P pili, S or F1C pili that attach the pathogen to host epithelia. The molecule FimH is part of the type-1 pili. In *in-vitro* assays it is necessary and sufficient to facilitate entry into epithelial cells of the bladder (reviewed by Mulvey 2002). Another group of human pathogenic bacteria for which adhesion constitutes the major virulence factor are the mycoplasmas. Mycoplasmas are wall-less bacteria; they have the smallest genome of self-replicating bacteria. *Mycoplasma pneumoniae* displays proteins of 169 and 30 kDa on the cell surface that appear to represent the adhesin and a protein associated with adherence, respectively. Additional proteins are necessary for adhesion (reviewed by Rottem 2003), for example, HMW1-HMW3 in *M. pneumoniae* (Dirksen et al. 1996). In some intracellular human pathogens, for example, *Listeria spp.*, *Salmonella spp.* or *Shigella spp.*, adhesion to the host cell is necessary before manipulation of the host cytoskeleton can be initiated that culminates in the internalisation of the pathogen (reviewed by Gruenheid and Finlay 2003 or Dersch 2003). In the three groups of human pathogens described above, specific adhesion is mediated by adhesins and receptors present on the host cell surface. An example is the protein that makes up the type-1 pili of uropathogenic *E. coli*. It interacts with mannose-containing glycoprotein receptors present on the surface of a variety of host cell types (reviewed by Dersch 2003).

5.1.2. Adhesion in human pathogenic fungi

Infections of humans with opportunistic microorganisms including fungi are frequent and on the rise as the number of immunocompromised individuals, for example, HIV infected patients or organ transplant patients having to undergo treatment with immunosuppressants are increasing (e.g. Hamilton et al. 1999 or Cotter and Kavanagh 2000). Important fungal pathogens fall within the genera *Aspergillus*, *Penicillium*, *Candida*, and *Blastocystis*. Superficial mycoses (infections with fungal pathogens) can become chronic as, for example, vaginal infection with *Candida albicans*; systemic mycoses can be lethal in patients with

deficiencies in their immune system as in the case of *C. albicans*, *Blastocystis dermatitidis* or *Penicillium marneffei* (Cotter and Kavanagh 2000, Brandhorst et al. 1999, Hamilton et al. 1999). Therefore, research on adhesion which is thought to precede invasion of host tissue is important to find control measures against fungal pathogens of humans. Fungal spores can be inhaled, adhere to the lung tissue, and invade the host from there or, as in the case of *Candida* species they can invade the epithelial cells in the vagina (Cotter and Kavanagh 2000). Some human pathogenic fungi have been shown to colonise contact lenses which serve as a reservoir for their infection (e.g. *Aspergillus niger*, Marqués-Calvo 2002). Many human pathogenic fungi are saprophytic, living in the soil on debris; some are able to infect immunocompetent humans and re-colonise the host when its immunity is compromised (reviewed by Klein 2000 for *B. dermatitidis*). Despite the long known importance of adhesion in these organisms relatively little is known about the molecular composition of the molecules involved in pathogen adherence. However, in *C. albicans*, several mechanisms for adhesion are known. As reviewed in Chaffin et al. (1998), both blastospores and filamentous forms of this dimorphic fungus show fimbriae 100 to 300 nm in length and approx. 5 nm in diameter. The fimbriae are made of heavily N- and O-glycosylated 8.64 kDa proteins. Glycosylation of the protein varies considerably as there are four species of glycoprotein with approx. molecular weights of 39, 47, 54, and 66 kDa. The fimbriae have been demonstrated to be important for the binding of the yeast to glycosphingolipid receptors on human epithelial cells (review by Chaffin et al. 1998). A group of molecules that has received much attention recently are adhesins. Adhesins are cell wall constituents of fungi that are implicated in the binding to host cells (Chaffin et al. 1998). Several adhesins of *C. albicans* have been cloned, sequenced, and their role in adhesion demonstrated (review by Sundstrom 1999). Integrin-like proteins have been found in *C. albicans* and the INT1 gene that is proposed to encode the approx. 188 kDa α-homologue has been cloned and sequenced (see Hostetter 1999). Gene disruption experiments demonstrated a decrease in adhesion in the mutants. Non-adherent yeasts were adhesive after transformation with the intact INT1 gene (Hostetter 1999). A gene family in *C. albicans* encoding agglutinin-like sequence (ALS) proteins has been identified. However, their role in adhesion has to be elucidated

(reviewed in Hoyer 2001). An important example of adhesins is the Hwp1 protein present in the cell walls of germ tubes of *C. albicans*. It constitutes a substrate for mammalian transglutaminase, an enzyme present on mammalian epithelial cells. The enzyme covalently cross-links the *Candida* cells displaying the substrate to surface components of epithelial cells in the mouth. Molecular mimicking of a substrate of a mammalian enzyme makes it easier for the pathogen to infest the host tissue (Staab et al. 1999). In *B. dermatitidis*, an adhesin termed WI-1 has been isolated from the surface of North American strains. The 120 kDa protein is involved in the binding of the yeast to macrophages which wild-type cells can invade. Targeted gene disruption and subsequent transformation of the mutant strain with the intact gene demonstrated a role of the WI-1 gene product in pathogen adhesion (Brandhorst et al. 1999).

5.1.3. Adhesion in plant pathogenic fungi

Adhesion is thought to be essential for successful infection of a new host plant (Nicholson and Epstein 1991). Apart from the advantage of making it more difficult for the fungal propagule to become dislodged, firm adhesion is believed to be important for signalling between host and pathogen (Nicholson 1996). From the observations that have been made so far, it has become apparent that different fungi use different attachment mechanisms (Nicholson and Epstein 1991, Mendgen and Deising 1993). Potentially adhesive cellular appendages termed fimbriae are known in many fungal species as described above for *C. albicans* (proposed by Gardiner and Day 1988). In other fungal species, these structures are up to 30 µm long and unbranched. The fimbriae of the smut fungus *Ustilago violacea* are made of a 74 kDa glycoprotein. Deglycosylation of the glycoprotein yields a 47 kDa portion that is proteinaceous (Celerin et al. 1995). Svircev et al. (1986) showed that antisera against fimbrial proteins react along the entire length of the fimbriae. They also demonstrate that the protein moiety is highly conserved amongst fungal species. The antisera even cross-react with the fibrils on the surface of ascomycetous yeasts. Antibodies towards the carbohydrate moiety are more specific (Gardiner et al. 1982).

Apart from the fimbriae a number of molecules involved in the initial attachment of fungal conidia have been proposed. In different fungi different adhesive molecules are found, most of them are carbohydrates, proteins or glycoproteins (Nicholson and Epstein 1991). Adhesive properties of these molecules can change with changing environmental conditions, for example, the temperature (Jones and Epstein 1989, Sela-Buurlage et al. 1991) or with the age of the cultures (Mercure et al 1994a). Nicholson (1996) proposed that all fungal adhesives must be water soluble for as long as they are inside the cell, and become water insoluble upon their secretion. Some fungi have vesicles in their spores that are lacking once the spores are settled. For these, a role in adhesion is suggested (Jones 1994). In these cases the secretion of the adhesive is a rapid process, not requiring protein synthesis, glycosyslation, and respiration. Some examples of what is known about adhesives of plant pathogenic fungi are given below. Although biochemical data are available for some adhesives found in propagules of plant pathogenic fungi, molecular data are thus far not available. Adhesives in the form of ECM associated with germ tubes of various fungi, for example, *U. viciae-fabae* have also been characterised (e.g. Beckett et al. 1990). Another example of the importance of ECM material in the adhesion of germlings has been found in *U. appendiculatus*. A decrease in adhesion levels was found in germlings that were grown in the presence of pronase E, a non-specific protease. Additionally, the pronase treatment had an impact in the directional growth and appressorium differentiation of these germlings when compared to the control, a finding that might indicate a role of the adhesive as a guidance molecule involved in fungal development (Epstein et al. 1987). Without treatment, the germlings adhered tenaciously to the leaf surface or parafilm, and only high levels of pronase E could remove them. The authors concluded that one or more adhesive proteins facilitate attachment. The level of adhesion of the germlings is also influenced by the surface hydrophobicity: it has been found that surfaces with similar hydrophobicity as bean leaves lead to the highest rate of adhesion of the germlings (Terhune and Hoch 1993). The importance of ECM material produced by *Uromyces* germlings has been recognised and studies have been undertaken to characterise the components of the ECM (Moloshok et al. 1993, Clement et al. 1993a). Interesting findings have been

obtained by Corrêa and Hoch (1995) who found 12 different RGD-binding proteins in microsomal fractions of germ tubes and proposed a role of these proteins in the interaction of the germ tubes with the plant (Corrêa et al. 1996).

Spore adhesion in true fungi

The ECM also seems to play a role in the conidial adhesion of some fungi. In *Blumeria graminis* f. sp. *hordei* the ECM material facilitates attachment of the conidia to their substratum immediately upon arrival (reviewed by Hardham 2001). However, in many other cases that have been examined to date, ECM material does not seem to be involved in spore attachment. In many cases, the adhesive is stored inside the spores and released upon arrival on the new host. In other cases, the adhesive is synthesised upon arrival and secreted as soon as it is produced. A few examples for either of those two strategies are given in the following paragraphs.

C. graminicola conidia require a hydrophobic surface for adhesion. The fact that adhesion occurs within min of contact probably means that an adhesive is stored within the spores and released upon contact with a host. Germination starts approx. 6 h after contact with the substratum and it is therefore believed that adhesion is essential for the development of the disease (Mercure et al. 1994b). Within 30 min of contact with the substratum the maximum of adhesion is reached and characterised by the attachment of 30-40% of conidia. It has been demonstrated that in the case of *C. graminicola* the maximum adhesion is influenced by conidium age but not by the concentration of the conidium suspension applied to the plant (Mercure et al. 1994a). For this process, respiration and transcription were not required, but glycoprotein transport and protein synthesis were necessary. Treatment of the conidia with the general proteinase, pronase E or with the lectin Concanavalin A (ConA) completely prevented adhesion. The latter finding implied that a glycoprotein is involved in adhesion (Mercure et al. 1995, Mercure et al. 1994a). In another species of *Colletotrichum*, *C. lindemuthianum*, a monoclonal antibody that reacts with a number of conidial surface polypeptides including a 110 kDa glycoprotein has been shown to inhibit spore adhesion to polystyrene (Hughes et al. 1999).

The rise blast fungus, *Magnaporthe grisea*, has spore tip mucilage (STM) stored in the conidial apex. The STM is stored in the periplasmic space between plasma membrane and cell wall and is released immediatly upon hydration. Within 20 min after hydration 90% of the conidia in the suspension are firmly attached to Teflon. In the case of *M. grisea*, adhesion is better on hydrophobic surfaces, for example, Teflon, compared to more hydrophilic surfaces, for example, glass. Conidia treated with ConA failed to attach, implying a glycoprotein is involved in adhesion (Hamer et al. 1988). The smo1 locus has been implicated in adhesion of *M. grisea* spores. However, the gene sequence is still unknown (Hamer et al. 1989). *M. grisea* has also been investigated regarding the presence of integrin-like molecules. Using antibody labelling, vitronectin-like and fibronectin-like proteins were found in infection-stage preparations. Upon addition of the antisera to conidial suspensions both adhesion and appressorium formation were adversely affected. Germination was not affected by this treatment. An additional clue for the involvement of the vitronectrin-like protein in spore adhesion was the localisation of the antigen predominantly to the STM containing conidial apex (reviewed by Dean 1997).

The obligate biotroph *Erysiphe graminis* needs a hydrophilic surface for adhesion (Nicholson et al. 1993). The conidia release a liquid that contains non-specific esterase and cutinase activity which leads to erosion of the cuticle exposed to the enzymes (Kunoh et al. 1988, Pascholati et al. 1992). The liquid is secreted in a two-step process, the first step being completed within 5 min and the second step after 10-15 min (Nicholson et al. 1993).

U. viciae-fabae conidia form an adhesion pad between the uredospores and the leaf surface. Deising et al. (1992) showed esterases and cutinase activity in the liquid. When autoclaved spores were tested, their attachment was much weaker. However, addition of esterases and cutinase isolated from conidia improved the rate of adhesion (Deising et al. 1992). Another mechanism of attachment of *U. viciae-fabae* conidia was proposed by Clement et al. (1993b). These authors observed increasing adhesion during 2 h incubation when the spores were placed on surfaces which would not be affected by cutinase or esterase activity. They explained the increase in adhesion by the increase in

the production of extracellular matrix material. Clement et al. (1994) also found that dry urediniospores initially adhere to a hydrophobic surface.

The conidia of *Cochliobolus heterostrophus* are dispersed by wind and splashing rain. They start germinating within a short time of arrival on the host. Just before the germ tube emerges the conidia start to adhere, approx. 20 min after inoculation. Within 1 h, over 90% of the conidia are firmly adhered to the host. The germ tubes are surrounded by ECM material and can penetrate the host within 3 h after inoculation (reviewed by Braun and Howard 1994). When the effect of surface hydrophobicity on attachment was tested, no correlation was found. *C. heterostrophus* conidia need respiration and protein synthesis for the production of adhesive material.

Jones and Epstein (1989 and 1990) have characterised the involvement of adhesion of *Nectria haematococca* in the infection process. They have found attachment to be a virulence factor (i.e. required for successful infection of the host). *N. haematococca* conidia reach maximum adhesion after 20 min, the hydrophobicity of the substratum not influencing the rate of attachment. Although adhesion is achieved within a short time, respiration and protein synthesis are necessary for production and secretion of the adhesive. A 90 kDa glycoprotein of the macroconidial tip mucilage (MTM) seems to be involved in adhesion. Within 1-2 h after incubation is started the adhesiveness of the macroconidia decreases. The 90-kDa glycoprotein is labelled by ConA and preincubation of conidia with either ConA or *Galanthus nivalis* lectin leads to a decrease in the level of adhesion (Kwon and Epstein 1997). However, adhesion-deficient mutants generated by Jones and Epstein (1990) also produce 90 kDa glycoprotein and MTM. It seems that additional factors are necessary for adhesion (Epstein et al. 1994). Conidia of adhesion-deficient mutants and wild-type were examined ultrastructurally. It was found that wild-type conidia produce a new outer cell wall layer. This layer was lacking in the adhesion-deficient mutants (Caesar-Ton That and Epstein 1991).

In *Aspergillus nidulans*, adhesion of conidia requires protein synthesis. Osherov and May (2000) examined temperature-sensitive mutants with blocked protein synthesis. They showed that these were unable to adhere or germinate. The same authors used differential hybridisation to detect transcripts for

potentially attachment molecules and found mucin-like transcripts that could serve that purpose (Osherov and May 2001). However, the function of the mucin-like molecules as adhesives still remains to be addressed.

In a variety of fungal species hydrophobins have been found. These are cysteine-rich low molecular weight proteins that are secreted and have been implicated in spore adhesion. Experiments on *M. grisea* have shown the presence of a hydrophobin encoding gene, mpg1 that is differentially expressed during conidiation and the early stages of infection. However, analysis of mpg1 deficient mutants demonstrated that conidia and germ tubes still adhere to Teflon (Talbot et al. 1996).

Spore adhesion in Oomycetes

In the oomycete species that have been examined so far, spore adhesion is a rapid process. In *P. palmivora*, the maximum level of adhesion is reached after 1 min after the induction of encystment (Bartnicki-Garcia and Sing 1987), in *P. cinnamomi* after 2 min (Gubler et al. 1989). However, encysting zoospores of these fungi only remain adhesive during the first 3-4 min. Cysts that have attached to a surface will remain attached, but unsettled spores are not able to attach themselves to their substrate after this time. So far, the mechanism by which the adhesive looses its adhesiveness is unknown. However, in *P. cinnamomi* increasing the calcium concentration increases the level of adhesion that is obtained (Gubler et al. 1989). As mentioned in chapter 1, a 220 kDa protein is stored in the ventral vesicles that are mainly located underneath the ridges of the groove of *P. cinnamomi* zoospores, is secreted upon encystment (Hardham and Gubler 1990). Several indications point towards the ventral vesicles as storage vesicles for the zoospore adhesive: firstly, the zoospores align themselves before encystment so their ventral surface is closest to the plant surface. Secondly, the contents of the ventral vesicles are secreted immediately upon encystment and at the same time the adhesiveness of the spore increases. Thirdly, the material seems to form a pad between host and cyst surface. In *Saprolegnia* zoospores, vesicles comparable to the ventral vesicles in *P. cinnamomi* have been found (Lehnen and Powell 1989). There, they are termed K-bodies and their contents have been implicated in zoospore

adhesion (Durso et al. 1993). In *S. ferax*, the K-bodies are labelled with wheat germ agglutinin and *Griffonia simplicifolia* lectin (Lehnen and Powell 1989).

The situation in *Saprolegnia* and *Phytophthora* species seem to differ from *Py. aphanidermatum*, where the adhesive might be stored in the large vesicles (Estrada-Garcia et al. 1990a). In *Py. aphanidermatum*, adhesion occurs within 2 min upon contacting the plant surface (Bartnicki-Garcia and Sing 1987). The adhesive appears to be a 200 kDa glycoprotein. Another molecule implicated in zoospore adhesion in *Py. aphanidermatum* is a 75 kDa protein on the zoospore surface that is recognised by a monoclonal antibody, PA-1. Interestingly, the addition of the antibody to zoospores induces encystment (Estrada-Garcia et al. 1990b), a finding that is comparable with monoclonal antibody Zf-1 that recognises the flagellar surface in *P. cinnamomi* zoospores (Hardham and Suzaki 1986, Hardham et al 1986).

As outlined above, adhesion plays a fundamental role for successful colonisation of a new host by pathogenic microorganisms. So far, either biochemical data of adhesives of fungal propagules or sequence data are available. However, in no case could the gene sequence for a putative adhesive be directly linked to a function of its product in adhesion. Monoclonal antibody Vsv-1 towards the Vsv protein, a *P. cinnamomi* protein that is secreted at the right time and at the right location to constitute an adhesive has been used unsuccessfully to screen an appropriate *P. cinnamomi* cDNA expression library (Dr. J. Marshall, personal communication). More monoclonal antibodies towards this antigen were generated in the work reported in chapter 4 of this thesis. Like Vsv-1, these antibodies recognise a proteinaceous epitope that makes them suitable for immunological screening of cDNA expression libraries. The current chapter was aimed at cloning and sequencing of the gene encoding the Vsv protein.

5.2 Materials and Methods

5.2.1. Immunological screening of *Phytophthora cinnamomi* cDNA expression libraries

Two cDNA expression libraries made from mRNA obtained from *P. cinnamomi* hyphae 4 h after induction of sporangium formation were screened by standard protocols: an oligo-dT primed library constructed in λZAPII and a randomly primed cDNA expression library constructed in λgt11 (Weerakoon et al. 1998, Marshall et al., 2001). Approx. 480000 phage were screened in the case of the λZAPII library and approx. 240000 in the case of the λgt11 library. The screening was carried out as described for the Pn14B7 antigen in section 2.2.2. In the case of λgt11, *E. coli* Y1090 cells were used as host cells and grown on LB agar. Purified monoclonal antibodies Pn-Vsv-1, Pn-Vsv-2, Pn-Vsv-3, and Pn-Vsv-4 were used at a concentration of 20 µg mL^{-1} each for immunolabelling. The strongly positive cDNA clone that was obtained was designated λgt11-Vsv and was screened repeatedly until plaque pure. It was grown on Petri dishes with a diameter of 150 mm as above, eluted into SM buffer and DNA isolated using standard protocols (Sambrook 1989). The insert of λgt11-Vsv was amplified by polymerase chain reaction (PCR) using primers flanking the insert, namely λgt11-3 and λgt11-4 (for primer sequences see Appendix VI). For this, 40 µM primer, 20 µM of each deoxy nucleotide phosphate, and 1.25 units of Taq polymerase in polymerase buffer were added to 1 µL phage stock. The PCR program was as follows: one cycle of 94°C for 30 s, 35 cycles at 94°C for 30 s, 60°C for 20 s, and 72°C for 90 s, and one cycle at 30°C for 60 s. The amplified insert was used to probe for matching phage in the genomic library and to probe genomic and plasmid DNA blots. Gel purification of the insert was carried out using commercial kits (e.g. QIAquick® Gel Extraction Kit, QIAGEN, Germany).

5.2.2. Design of custom primers and DNA sequencing

Design of custom primers and DNA sequencing was carried out as described in sections 2.2.3 and 2.2.4. Primer λgt11-3 or λgt11-4 was used for sequencing of the cDNA clone (1.6 µM per sequencing reaction). Primers T3 and T7 (New England BioLabs), and custom primers were used to sequence sub-clones of the genomic clone. For a list of primers and their sequence see Appendix VI.

5.2.3. Screening of a genomic library

A genomic library of *P. cinnamomi* constructed in EMBL3 was screened under high stringency conditions as described by Marshall et al. (2001). For production of the library see Weerakoon et al. (1998). A DNA probe containing the insert of the cDNA clone λgt11-Vsv was radiolabelled with [α-^{32}P]dCTP with a Megaprime random-oligonucleotide DNA-labelling kit (Amersham Pharmacia Biotech). Again, the positive clones were re-screened until plaque pure. The phage were grown on agar plates and eluted into SM buffer containing chloroform. DNA was extracted using the Qiagen Lambda Midi kit (QIAGEN, Germany).

5.2.4. Subcloning of a genomic clone (EMBL3) into the bacterial vector pBluescript

DNA fragments resulting from a restriction digest of the positive genomic clone with the enzymes *Sal*I, *Sac*I, and *Xho*I recognised by the λgt11-Vsv cDNA clone were subcloned into the bacterial vector pBluescript. *Sal*I fragments were shotgun cloned into pBluescript, the other two fragments were gel purified after gel electrophoresis and cloned. *E. coli* DH5α cells were transformed with the resulting plasmids and glycerol stocks made using standard procedures (Hanahan 1985, Sambrook 1989). Plasmid DNA was extracted using a commercial kit (QIAprep® Spin Miniprep Kit, 27106, QIAGEN, Germany).

5.2.5. Southern blot analysis of the *Vsv* gene

For genomic Southern blot analysis, 20 µg DNA per lane were digested using restriction enzymes *Sal*I, *Eco*RI, *Bam*HI, and *Sac*I according to the manufacturer's recommendations in a final volume of 30 µL and incubated for 2 h. For Southern blot analysis of the genomic clone and the subclones, 200 ng were used. The fragments were then separated on a 0.8% agarose gel in Tris-acetate/EDTA electrophoresis (TAE) buffer (1 mM EDTA in 40 mM Tris-acetate), stained with ethidium bromide, treated for 10 min with 2% HCl, for 15 min with denaturing solution (1.5 M NaCl and 500 mM NaOH), and for 15 min with neutralising solution (1.5 M NaCl in 500 mM Tris-HCl pH 8.0) before overnight capillary transfer in 20xSSC (3 M NaCl and 300 mM sodium citrate) onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech). The membrane was rinsed for 2 min in 2xSSC and the DNA was fixed onto the membrane by microwaving the blot on the highest setting for 2 min. The blot was wetted with 2xSSC, pre-hybridised, and hybridised as described in section 5.2.3. A phosphoimager screen was exposed for 5 s (some of the subclone Southern blots) to overnight (genomic Southern blots) to the blots and scanned on the following day using the ImageQuant 5.0 software (Molecular Dynamics).

5.2.6. Nucleotide and amino acid sequence searches and analyses

The non-redundant database was searched through the NCBI and the *P. sojae* and *Thalassiosira pseudonana* genome database was searched on the official website using the BLAST programs BLASTN, BLASTX, and TBLASTX. The nucleotide sequence was searched for introns using the software program FGENESH (http://www.softberry.com/berry.phtml?topic=fgenesh&group=programs&subgroup=gfind). It was translated into amino acid sequence using Pepdata on the WebANGIS (see section 2.2.4). The molecular weight and theoretical isoelectric point were calculated with protparam on the ExPasy server (Bachmair et al. 1986, Gonda et al. 1989, Tobias et al. 1991, Ciechanover and Schwartz 1989; http://kr.expasy.org/tools/protparam.html). The inferred amino acid sequence was searched for a signal peptide using the

SignalP software program (Nielsen et al. 1997a, Nielsen et al. 1999, Nielsen et al. 1997b; http://www.cbs.dtu.dk/services/ SignalP/). For internal repeats the first 900 amino acid residues were searched with radar (http://www.ebi.ac.uk/Radar/) and domain searches with the SMART program were carried out on the entire putative Vsv protein sequence (see section 2.2.4). The entire inferred amino acid sequence of the protein was also examined visually to identify internal repeats. The amino acid residues of the internal repeats were compared to a consensus sequence given by Adams and Tucker (2000). Additionally, all other amino acid residues in the repeats were visually examined and recognised as conserved within the proposed Vsv protein if they occurred in 22 or more of the repeats.

5.3 Results

5.3.1. Monoclonal antibodies identified a positive cDNA clone

Immunological screening of 480000 phage of a λZAPII library made from mRNA isolated from sporulating hyphae with a mixture of monoclonal antibodies Pn-Vsv-1 to Pn-Vsv-4 yielded no positive phage. Analogous screening of a λgt11 library of the same developmental stage lead to the identification and purification of one strongly and two weakly positive phage. The strongly positive cDNA clone was designated λgt11-Vsv. Amplification of the cDNA insert with PCR yielded a DNA fragment of approx. 550 bp that was gel purified (Fig. 5.1). The cDNA insert was used to probe a genomic DNA blot and to obtain sequence information about the cDNA clone (see Fig.5.5 for the full *Vsv* sequence). An open reading frame extended in both directions from the cDNA clone and therefore, a genomic library was screened with the cDNA insert as a probe in order to obtain the full sequence of the putative *Vsv* gene.

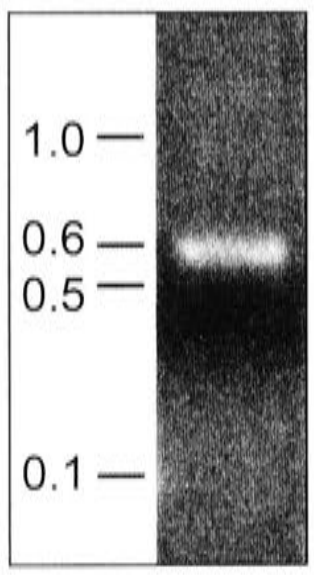

Fig. 5.1 A product of approx. 550 bp is obtained by polymerase chain reaction of the insert of cDNA clone λgt11-Vsv. The amplified insert of λgt11-Vsv is shown after agarose gel electrophoresis and staining with ethidium bromide. Numbers on the left indicate size in kb.

5.3.2. Subcloning of a genomic clone and sequencing of the *Vsv* gene

The λgt11-Vsv insert was used to screen a genomic *P. cinnamomi* library constructed in EMBL3. A positive clone was purified in several rounds of screening and was designated EMBL3-Vsv. DNA digests and subsequent Southern blot analysis were used to characterise the clone (not shown). The restriction enzyme *Sal*I was used to cut out the insert. Southern blot analysis of the *Sal*I digest revealed a fragment of approx. 3-3.5 kbp that was recognised by the λgt11-Vsv insert (Fig. 5.2).

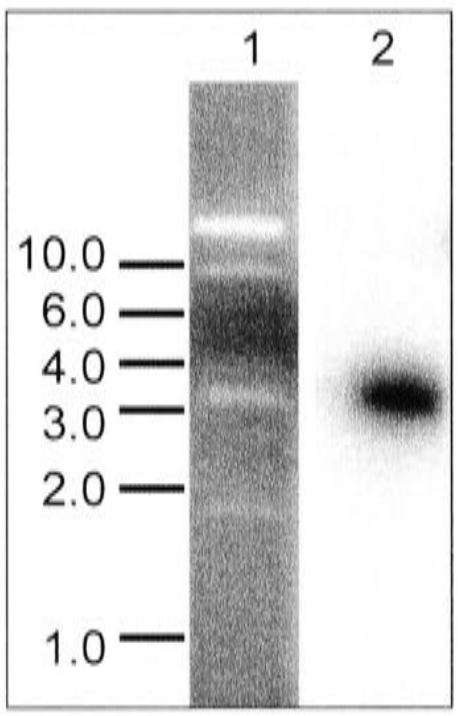

Fig. 5.2 Genomic clone EMBL3-Vsv after restriction digest with *Sal*I. Lane 1 shows the fragments after agarose gel electrophoresis, lane 2 the results after Southern blotting and probing with the insert of cDNA clone λgt11-Vsv. Numbers on the left indicate size in kb.

Restriction digests with *Sac*I or *Xho*I yielded fragments of approx. 7-8 kbp that were positive when probed with the cDNA insert. The recognised *Sal*I, *Sac*I, and *Xho*I fragments were subcloned into pBluescript, analysed using restriction enzymes and Southern blotting (e.g. *Sac*I and *Xho*I fragments, Fig. 5.3), and sequencing in both directions carried out.

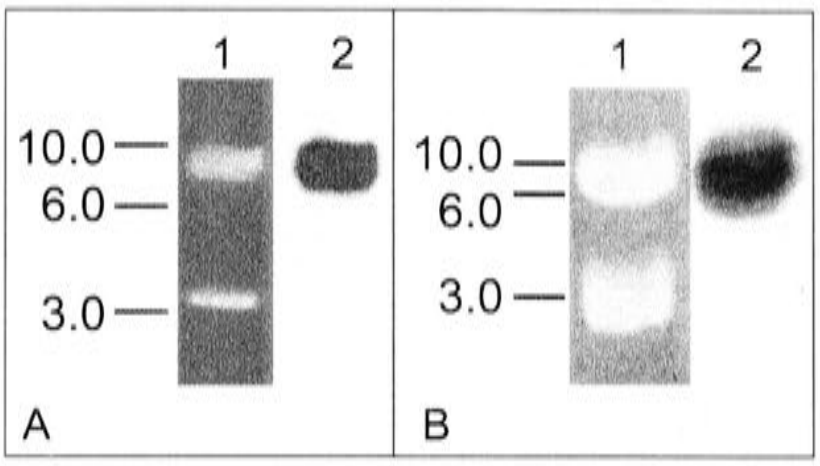

Fig.5.3 Subclones containing *Sac*I (A) or *Xho*I (B) fragments of genomic clone EMBL3-Vsv after restriction digest with *Sac*I (A) or *Xho*I (B). In each case two fragments are obtained (lanes 1 and 3): the fragments on the top are recognised by the cDNA clone λgt11-Vsv insert after Southern blotting (lanes 2 and 4), the fragments at approx. 3.0 kb represent the vector. Numbers on the left indicate size in kb.

An open reading frame of 7356 nucleotides was identified. Figure 5.4 shows the relative orientation of the genomic clone, cDNA clone, and subclones to each other as well as key restriction sites. In Figure 5.5 the partial sequence of the genomic clone including the open reading frame of the putative *Vsv* gene is given. The region upstream of the putative *Vsv* gene showed no *Phytophthora* transcription initiation site known so far when compared to the consensus promoter site found in the *P. infestans* gene cluster IpiO and IpiB (Pieterse et al. 1994).

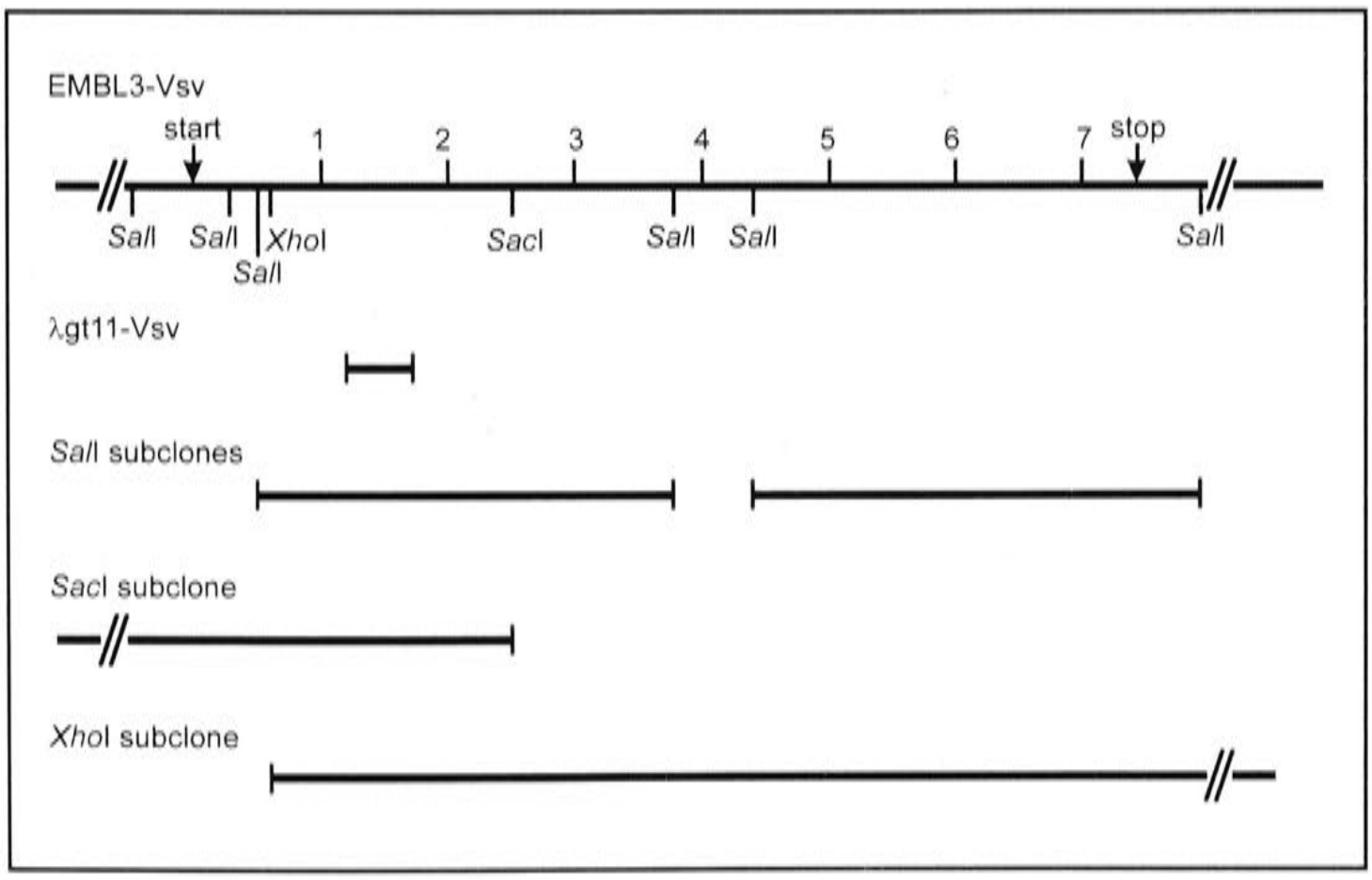

Fig. 5.4 Diagram showing the relative position of cDNA clone λgt11-Vsv and four subclones on the genomic clone EMBL3-Vsv. Start and stop codons are marked by arrows on clone EMBL3-Vsv. Numbers on the line representing EMBL3-Vsv represent length of the gene in kb.

```
                    AACC TTGCAGCCGC TTTGGTCCTC GCACTGCCCG

CTTGATGACG TTCGCGAGCA ACATCTTAGA ACACCCCGAG TCCTCGTAGT -701

AGGCGCCCAC ACTGTCGGTG CCAGCTGCAG TGTTGATCAT CATGATAGCC

AAGCCAGCAG CGGCAGGTCG CAGGATCTTC ATGTTGAGCT TGGCAGGGCT -601

GATGGGCGAA GAACGGGCTT GTGTGGCTGC GGTTTGGATA CTGAGCCTTG

GACCGGAGTG GGAAGACGGC AAGGACGAAG GACGAGGTGA CTTTGTCGAC -501
                                                 SalI
GTTACGCCTC CCCTCCGAGT CTTGTTTGCC GAGCCGTTGA GGGCCTGAAC

TGGAGGCGCA TGGGGGTGCG TGGCGCTATT CCTGTGCATT AGCGTCAACG -401

TCATTTACAG GCAACAATCA CTCCGACCTA CTTCAACTTG ACAATCACTC
                    Forw-01
ATGCCTGTCA AAAATGCGCT GAAGATCGCA CCCTTTCGTG GGCGACTGCT -301

CAGGGGCAAA ATCTTTGCGA ACGACCGTTC ATGCTTGTCA TCGGTGGCTT

TGACCAGCAA GACAAACACT CATGCCTATC ATCGGACGGT TGGCACGCAG -201
                    Rev-12
CGGAGGAAAA GATCGCGCTT TGACAAGCAT CAAGGACCCG AGGTGCAAGG

CTCCGTCGCC AGTGGCCTCA TTCCCGAAAA CGCCGAGTTC GAGTCACGTC -101

CAAGGCTCTC AGCGGTCAAC AACAAGTCCC CCGCAGTGTC CCCAACGTCA
          Forw-02
ATTCAAGTCG AGTCGAGCCA ACTGTCAACC TGACACCCGC AGCGTGAGGC

ATGCACCTGG ACCCCTTCCG CCTCGCCGCC TTGGCGCTGG CGCTGATGAC

CTCGTCGCGC GGTGCTTCAG CTGAAGGGGA CACCTTCTGG GGGCCCCTTG  100

GAGGGACCTC CGCCTATGGC ACCTCCGCCT ATGGCACCTC AGCATATGGC

ACCTCAGCCT ATGGCACCTC CGGCACCTCT GCTTATGGCA CCTCCGGCAC  200

CTCTGCCTAT GGAGCCTACG GGGGGTTTAA CGTCGGCGGC ACTGTGTCCA
                                           Rev-11
GCATCATCCA GAATGTCCTT CCTCCAGTCG ACTGCCAAGT GGGGCCCTGG  300
                          SalI
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv surrounding and including the putative *Vsv* sequence. The start codon is printed in bold, the stop codon marked by an asterisk. The sequence of the cDNA clone λgt11-Vsv is printed in italics. Deviations of the cDNA sequence from the sequence of the genomic clone are given below the genomic sequence. *Sal*I, *Sac*I and *Xho*I restriction sites are marked. Primer positions are underlined and primer names given below their sequence. Forward primers are named Forw-01 to Forw-18, reverse primers Rev-01 to Rev-12. Additional primers used to sequence subclones were T3 and T7 primers.

```
AATGACTACG TCGGCTGCTA TTCCGCCTAC TCCTCCACCA AGACTCGCCG
                      Forw-03
TCGGTCTGTG ATTGTCTGGC CCCTCAACAA CGGAGCGGCC TGCCCTGCCC   400

TCGAAGACAC TCAGCCGTGC ATGCCCCAAG ACTGCCAAGT CGGCCCTTGG

TCCGCCTACA CCCAGTGCGA TGCGTTGTGC GGCAAGAAGA CCCGCACACG   500

CGCCGTGATT CTGCCGGCCC AAGACGGAGG CGCGGCCTGT CCGTCACTAG

TCGACACGGC TCCCTGCGAC CCCATCAACT GCGTTGTGAG TGACTGGTCT   600
SalI                              Rev-10
CCGTGGAGCA CCTGCCTGCT GGCCAAGAAG ACTCGCTCGA GATACGTGCA
                                  XhoI
GGTCTGGTCC CAGTACGGCG GCACGCCCTG TCCGGCGAAC CTGCTCGACG   700

TGCAGCCTTG CACCCCGGTG GACTGCGCTG TGACTGATTG GAGCCCGTGG

AAGATCATCG GTCTCAAGAA GACGCGATCC CGGGACATCA CCTGTCCGGC   800

TGATGGGGGC AAACCCTGTC CAGCGTTGGT CGAGGAGGCG GTGTGCAACC

CCGTCGATTG CGTGGTGAGC GCGTGGTCCT GGAGCGGATC GTGTGACCCC   900

ACTACGAAGC TCCGAACACG AACCCGAGGC ATCGTCACCG CCGCTCAAGA
    Forw-04
CGGCGGCAAG GACTGCCCTG CTCTGCAGGA GACCGTGACG TGCATCGACT  1000

GGGTGGTGAG TGCCTGGAGC GGCTTCTCCG TCTGCAACCC GCTCACGGGC

ACCAAGACGC GAACGCGGAC GGTGGTCACG GCAGCGGTCA ACAATGGAGC  1100
                                   Rev-09
TGACTGCCCG ACGCTCGTGG ATACGACGCC GTGTGATCCA GTGCCCTGTG

TGGTTGGCAA CTGGACGTCG TGGGGTTCCT GCGACTCGAA GACGTTGCAG  1200

CGCCAACGCA CTCGCGTTGT GGTGACGACG CCGTGCTACG GAGGAGCAGC

CTGCCCCCCG CTCGTTGACA CTCAAGCTTG TGTGCCTCAA GACTGCACTG  1300
                                   Forw-05
TCAGCGACTG GAGCCCTTAC GAATACTACG TCAAGAATAC GGACGGGCTG
          Rev-08
CGCTACAAGC GGCGCTCGCG GACGGTGATC CAAGCTTGCG ACGGCGGTGC  1400

AGCGTGTCCA CCGCTGCAGG AAGAGGTGCA GTACCAGCCA GTGGACTGCA
                                   A
AAGTGAGCAA GTGGGTTTGG GACGCCAACG GAGCTGGAGC GTGCCCTCCC  1500
           A
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv – continued

```
GGAACCCAGC GCACACGCAC TCGCACGGTT GTAACCCAAT CACAGGATGG
                                          C
CGGTGCGGCT TGTCCGGCGT TAGTGGAGCT GGTGGCTTGT GGTGATTGCC 1600

AAGTGAGTGC CTGGAGCGCG TTCGGAGCCT GCGACGCCGG AACCGTGACC

CGCAGACGAA CTCGAACTAT CACGCTGCAG CCTATTGGCG ACGGAGCTGC 1700
A           Forw-06
CTGCCCAGTA CTTGAGGATA TCGCTGCGTG CGAGCCTATT CCCTGCCAAG

CGAGCGATTG GACTGTCTGG GGCCCCTGCA CAGCCTCCAG TATTCGCCAA 1800

CGCACCCGAA CTATCGTCAC CCCTGCCAAG TACGGCGGCA CCGACTGTCC

GCCGCTCGTG GACCAGCAGA ATTGCAACCC AGTTGACTGC CAGGTGAGTC 1900

CTTGGGGTCC CTACGAGCTC CTTGGCGCGA CAAAATGCCG CCGACGAACG

GTTCTACGCG ATGCCGACGG TGGCAAAGCA TGTCCTGCTC TTGTGGAAAC 2000

GACGACGTGT AAAAAGGTGG ACTGCGTGGT GGGCGACTGG GGGTCTTACT

CGGCTTGCAA CCCCATCACC AAGCAACGCT CTCGCTCCCG TGTGGTCGTC 2100

ACTTCCCCTC AAGACGGGGG TGCAGCATGT CCAGCTTTGA TCGAGTCGGT

AGCCTGCGCT CCTGTCAATT GCCAGGTCAG TAGCTGGGGC ACTTGGAGCG 2200
              Forw-07
ACTGCTCTCC GATCACCGGC AAACGCACGC GTCGCCGCAC TGTGACGACG

CCCTGTGCCT ACGGAGGCAC CGACTGCCCG CCGCTTATCG ACGAGGACAA 2300

GTGCCAACCT GTGGACTGCA AGGTCAGCGA CTGGACGCTG TGGAGCGGAT

GCAACCCGCT CACCAGGACC CGCTCGCGCA TTCGCTCGCT GATCACCCCA 2400

GCCAGCTACG GCGGAGCGGC GTGTCCAAAC CTTCTGGAGA CTCAGGTTTG

CGTGCCGACG GACTGCATCG TGAGCTCCTG GTCGCCCTTC ACGTCCTGTG 2500
                        SacI
ACAACAGTAA CAACGGCAGC GGCAAAGAGC GTCGATTCCG AACGGTGCTG
        Forw-08
CAGCAGCCCG ACGGTGGAGC TGCGTGCCCG CCACTGATGG AGGAGCGGCC 2600

AGCGCCCAAG GTGAACTGTG TTGTGAGTGA CTGGAGTGAC TGGGGAGTGT

GCGACCCCGT CCTGTTCGTC CGCGTGCACA ACCGATCGGT GATCACCGAA 2700
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv – continued

```
GCGAAGAACG GCGGCAGGTG CTGCCCAGCG TTGCAGGGAA CGCAGCCCTG

CTGTCCCAAG CGCGTAGACT GCAAGGTTGG GGCCTGGTCG GACTACCCGA 2800

CTTGTGACCC AGCTTCCAAC ACTCAAACAC GCACTCGCAA TGTGACTCAA
                                    Forw-09
GCCGCTGGTC CTGGTGGTAG AGCTTGTCCC GATCTCCAGC AAACTCGCAG 2900

CTGTACTCCG AGCGTGGACT GCCAGGTCGG AGACTGGAGC GATTGGGGAG

GCTGCTCCAA GAGCAAATTG GTGCGCACTC GAACACGTCA GGTCACCGTC 3000

CCGCGAACGG GCAAGGGGGC CAAGTGTCCA GCACTGAAGG ACACGCAGAG

CTGCATCCCT GTGGACTGCA CCGTGAGCGA CTGGGGAGAC TGGAGCGCCT 3100

GCGACTCGGT CGCCGGCCTC AAGACACGGA CCCGAACTGT TTTGCAAGAT

GCCGACGGCG GCAAGAAGTG TCCGAGCCTC ACGCAAACGA AGACGTGCAC 3200

CACCAACGTG GACTGTCAGG TCTCGTGCTG GGGCGACTAC GGAGTTTGCG

ATGAGTCGGT CTGGAAGCGC ACCAAGACTC GGTCGATCCT CGTGCAGCCG 3300

AAAGGCAAGG GCAAAGCGTG TCCCTCGCTC ACACAGACGG ATGCGTGCCC

ACCACGAGAC TGCAAGGTGA GCGGGTGGTC TCAGTACGGA GCCTGCGACG 3400

CCAACGGGAA CCGCAATCGC ACTCGACAAA TCGTGACTGA CGTCCGCGGC

GGAGGCAAAG CGTGTCCGCC ACTCCAGGAG ACCGGTGTTT GCAGCGCCGT 3500

CAACTGTGGC GTGACAGCTT GGGGCACTTG GAGCGACTGT GACGCCACTT

CCAACATGCA AACGAGATCT CGATCGATCC AAGTGCAGCC TGCGTACGGC 3600

GGTACAGCTT GTCCACCGCT CACGCAGACC CGAAGCTGCC AGAAGTGCAT
                                    Forw-10
CGTGAGCGAC TGGAGCGACT GGTCCATCTG CACGGCGGAC ACGCCCACGC 3700

GATTCCGAAC GCGGGTGGTT GTTCAGCAGC CCCCGCCTCC GTCGACTTGC
                                    SalI
GGCAATTCTG ACACTGACGA CGTTGCGGTG AGTTTGCAGT GCCCTGTGCT 3800

GAGGGACGTG CAGCCGTGTG ATGCCATCAA CTGCACCGTG ACTGATTGGG

GTGACTGGGG CGACTGCAAT CCCGATACCT ACGTGAAGAC TCGCAACCGC 3900
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv – continued

```
ACCATCGTGC AGCCCGCAGT GCGTGGCGGC ACCGATTGCC CCGACCTCAC
GGACACTGCT GTCTGCCCCT CTGTGGATTG TGTCATGGGG GACTGGACGG 4000
ACTGGAGCCC GTGCGGACTG AACGACCTCA TGTCGTCGCG TTCACGTCAA
GTGCTGGTGC CTGCTGCATT TGGAGGCGCC GAGTGCCCGT GGCAAATGGA 4100
GGGCGTCGAC TGCCCGTCCA TTGACTGCGT TCTCAGTGAC TGGACGGACT
     SalI          forw-11
GGAGCATCTG CACGGACGAC ACACCGACAC AGACTCGCGA GCGCTCAATT 4200
GTCCAGGATG CTGTTCGAGG AGGAGCTGCG TGTGACGTTT TGGCGCAGTC
TCGCACGTGT CCTCCCGTGA ACTGTGAACT GAGCGACTGG AAGGCGTGGC 4300
AGGTGTGTGA TGCCAAGACG GGAACGAAAA CGCGCACGCG GGACATCACA
       Rev-07
CGATCTCCTC AGCGCAACGG AACGGCCTGC GACGCGCTTC AAGACATCCA 4400
GCCTTGTGAC GCTGTGGATT GCATCGTCGA CCCGAACTGG GGCGACTGGA
                          SalI
GCGCGTGCGA TAACACCTGC GGCAAGCGCT ACAAGAACCG ACAAATCCTG 4500
CAGAACCCGC TGTATGGCGG CGTTGCTTGT GCTGCTCTGG TGATGGAGGG
CCCCTGCGAG CCTGTGAATT GCGCCGTGTC CGCGTGGAGT GACTGGAGCG 4600
                                   Forw-12
ACTGTGATGC ATCTACTGGC ATGCGCACGC AGACTCGCAA CGTCACGCAG
CAGGCTTTGT ATGGCGGTTT GCCGTGTCCT GCGCTGGTCC AGCAGAAGCC 4700
CTGTGATCCA GTGTGCTGCA CTGTGAGTGA GTGGTCTCCG TGGACATCCT
GCGATACCGA CGACCCACAG CAGCACCGCT ACCGAAATGT GACGCAACAG 4800
CCGCTCTATG GCGGCACTCC GTGTCCTGAC CTCACGCAGG ACGTAACTTG
TCCTCCGATC AACTGCGCTG TCGGACTGTG GAGCGACTAC GGGCTCTGCG 4900
       Rev-06
ACGCTAGTTC AGGTCTGCGT ACTCGCACAC GCTCGGCTAC ACAGGTACCT
CGACATGGAG GCGCTGACTG CCCAGCTCTC AACAGCACCA TCGCCTGTGA 5000
                          Forw-13
TCCAGTGGAC TGCAAGGTCA GTGACTGGAG CGACTATGGC GCGTGCGACG
CCGTGACGTT CAAGAAGACG AAGACTCGCA CGGTGGTGCA GCAGCCGCTT 5100
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv – continued

```
TACAACGGTG CGCCTTGCCC CGATCTGACC AACGACTTGC TGTGTGATCC

GATTCACTGC GCCGTGGGCC CGTGGGGGGC TTGGGAGAGC TGCGACGCTA 5200

CATCGCAAAC CAAAACGCGT CGGCGAACCA TCACTACGCA GCCGAAGTAT

GGCGGGTGTG CGTGTCCAGC ATTGACCGAT TCGGCGGCTT GCGATCCGGT 5300

GAACTGCGTC ATGGGACCCT GGTCTGACTG GAGCATTTGC AATGCTGACG
                        Rev-05
GCACCGACAC CAGCTCATTC CGAACGCGGT CGGTGGTGCA GCCTGCGCTT 5400

TATGGCGGCT CTGCATGTGG AGCCACTCGC GAAGAAGTGC CATGTGGAGC

TGTGAACTGC GTCGTGGGCA ACTGGACGGG CTGGTCGAGC TGCGACGCAA 5500

GCTGCGGCAA GAAGTCTCGC ACTCGTCAAG TCACGCAGCG CGCGTTGTAC
          Forw-14
GGAGGAGCGG CATGTCCTGC TCTAGAGGAC CTCGCGAATT GCGATGCAGT 5600

GGACTGCGTG GTGGGCGACT GGGATGCGAC GTGGTCGGCT TGTGACTCGA

ACTCGGGGCT CCGTACGCTG ACTCGTCCAG TGCTACAGGC AGCGCTCTAC 5700

GGCGGCAAGG CTTGCCCCGT GACCATCAAG ACCAAGTCGT GTGACCCGGT

TGACTGTACT GTGAACGACT GGCAGAGTTG GGGTGTCTGC AACGCTGATA 5800

CTGGGAAAAA GACACGCGTG CGCACTGTCA AGCAGGGCGA CCTGTATGGA
                                    Rev-04
GGATGCAAGT GCCCCGACCT CACTCAGGAC GCACCCTGCG ACCCTGTTCC 5900

GTGTACCGTT GGGCCTTTCA GTGACTGGAC AAACTGCACA GACGCCGATG

CAACCACGTC GCGCACTCGG CCGATCACGC AGATTGCTCT CTACGGCGGT 6000
                        Forw-15
GATGCTTGCC CCTCGCTCAC GGAGACTGCC GCCTGTCCTC CTGTGGACTG

CGTGCTGGAT TCGTGGGGAC CTTGGAGTGA CTACGACGCT GTCACTGGAG 6100

TCCGTACGAG AACTCGAACT GTCCTCCAGC CGTCGGTGCG TGGTGGATCG

CCGTGCGACG TCACCTTGGA CTCGCAGACT GCGCCGCCTG TGAACTGCCA 6200

AATGGGCAAC TGGACCGAGT TCGGCCCGTG CAACGACGTC GCCGGGGTCA

AGACTCGGTC TCGCCCGATA ATCGTTGCTC CGAAGTACGG CGGACTCGCA 6300
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv – continued

```
TGTCCTGATC AGTTCCAGAA CACGACCTGC GACGCGGTGA ATTGTGTGGT
                                                Rev-03
GAGTGAGTGG AGTAGCTGGG GAGCGTGCGA TTCAAACACC GGCAACAAGA 6400
      Rev-03
CTCGCCGTCG CGATATCCTG CAGAAGGACA AGTACGGTGG CGCCTGCTGC
                          Forw-16
CCGACACTCA CAGACTCGGC TCCTTGCGAT CGCGTGGACT GCGTCATGAA 6500

CGACTTCGGC CCGTGGACGT CCTGTGACGC TACAACTGGG GCCAAGACGA

GAACGCGCAC GGTTCAAGTC GCGCCGTTGT ACGGAGGAAG TGCGTGTCCG 6600

AGCACTACGG AGCAGGGATT CTGCAACCCC ATCGACTGCA AAGTCAGTGA

CTGGAGCGCT TTCGGAGCTT GCAACACTAC ATCGGGACTG AAGACTCGCA 6700
                          Rev-02
CGCGCACGGT GACCACGCAA CCGCTTTACG GTGGAGCAGC GTGTTTGCCT

CTCACAGATT CGGCGGCCTG CGATCCTGTC AACTGCCAGG TTGGCGCGTA 6800

TGGCTCGTGG TCGGCATGCA GCGCAGCGAC ACTGACCTCG ACGCGTACGC

GAAAGATCAT CGTCAGTCCG GCGTACGGCG GCATCGATTG TCCGTGCTTG 6900

ACTGAGACGT CCCCGTGCAA GATGCCAACG CCGACGAACT GCACAGTTAC

TGCGTGGTCG GACTGGACCA AGTGCTCTAG CCGGAGCGGC ACGAGGACGC 7000
                                                Forw-17
ATACTCGCAA AGTGGTGACG GCAGCGACCA ACGGAGGCAC GGCATGTCCT

GCATTGAGTG AGACCGGTTC GTGTGCTGGG CTGACGTGCA CGATGGGCGC 7100
      Forw-18
TTGGTCGAGC TGGACGACTG GCTGCGATAG TTACGGCATG CAGACCCGAA
                          Rev-01
CGCGCGTGAT TCTGGATGAC CCATACACGT GCAGCGACTT CTGCGAAGCT 7200

ACTATGGACT GGCGCGCGTG CACCTACAGC AACTCGACCA ACGACTACTC

CCCTCCTGCG GCGAGCCTCC TCCTCGCTAG TGGTGAGATG GCTCTGGAAG 7300

CTCAGGCGTT TGATGAAGAT GGAGACCCAA GCCACGTACA GGCATTGTAT

GTTTAGTTTG CATGTTTGCT TCGACCAAAG TGTAGCGTGC CGATCGCTTT 7400
    *
ACCATTTCTT TTCTCTCTCG CTACCTACTT ACCCGTTCAA CATATTTTTG

TGAAATGTGA TCCGACAAGA GGAAGGTCAT GATAGTAGAA TACTCCGTTT 7500

TACTACGTAG TAGGGCTTTC GTCGCATGCG ACTACAAAGT CGAC
```

Fig. 5.5 Nucleotide sequence obtained from genomic clone EMBL3-Vsv - continued

Nucleotide-nucleotide BLAST (BLASTN) searches of the *P. sojae* genome database yielded multiple hits of 120 to 480 nucleotides along the entire length of the gene with identities of over 85%. BLASTN searches of the *Vsv* gene sequence did not reveal significant homologies in the non-redundant database at NCBI or the database of the *T. pseudonana* genome. However, BLAST searches of the non-redundant database with the partial sequence obtained from the *Sac*I genomic subclone approx. 7 kbp upstream the putative *Vsv* gene revealed high similarity to a necrosis inducing peptide in *P. infestans*, *P. parasitica*, *P. sojae*, and *Py. aphanidermatum* (data not shown).

5.3.3. Inferred amino acid sequence encoded by the putative *Vsv* gene, similarity searches, and sequence analysis

The nucleotide sequence of the putative *Vsv* gene was translated into amino acid sequence (frame +2; Fig. 5.6). The software program protparam predicted a molecular weight of 261.6 kDa and a theoretical isoelectric point of 5.52. A 25 amino acid residue leader sequence marking the putative protein for secretion was predicted by SignalP. The first 900 residues of the putative protein were searched for internal *de-novo* repeats with the program radar and thrombospondin type 1 (TS1) repeats were identified. The SMART program predicted a signal peptide of 25 amino acids, a region of low complexity, 44 TS1 repeats, and a carboxy terminal domain. However, closer examination by eye revealed 47 instead of 44 TS1-like repeats. A schematic representation of the putative Vsv protein is given in Fig. 5.7. The amino acid sequence was compared to the TS1 repeat consensus sequence described by Adams and Tucker (2000). A TS1-like motif of 45 to 55 amino acids in length containing the consensus amino acid residues W8, S9, W11, C14, R25, R27, C41, C51, and C56 was identified. Cysteine residues C18 and C29 are conserved in the classic TS1 repeat. They are not present in the TS1-like repeats of the putative Vsv protein. In addition to the nine amino acid residues that are conserved in TS1 repeats, amino acid residues V5, S6, D15, K23, T24, V29, G37, G38, P42, L44, P53, and V54 occur in 22 or more of the 47 TS1-like repeats in the Vsv protein. Fig. 5.8A shows an alignment of two TS1-like repeats of the putative

```
   1  MHLDPFRLAA LALALMTSSR GASAEGDTFW GPLGGTSAYG TSAYGTSAYG
  51  TSAYGTSGTS AYGTSGTSAY GAYGGFNVGG TVSSIIQNVL PPVDCQVGPW
 101  NDYVGCYSAY SSTKTRRRSV IVWPLNNGAA CPALEDTQPC MPQDCQVGPW
 151  SAYTQCDALC GKKTRTRAVI LPAQDGGAAC PSLVDTAPCD PINCVVSDWS
 201  PWSTCLLAKK TRSRYVQVWS QYGGTPCPAN LLDVQPCTPV DCAVTDWSPW
 251  KIIGLKKTRS RDITCPADGG KPCPALVEEA VCNPVDCVVS AWSWSGSCDP
 301  TTKLRTRTRG IVTAAQDGGK DCPALQETVT CIDWVVSAWS GFSVCNPLTG
 351  TKTRTRTVVT AAVNNGADCP TLVDTTPCDP VPCVVGNWTS WGSCDSKTLQ
 401  RQRTRVVVTT PCYGGAACPP LVDTQACVPQ DCTVSDWSPY EYYVKNTDGL
 451  RYKRRSRTVI QACDGGAACP PLQEEVQYQP VDCKVSKWVW DANGAGACPP
 501  GTQRTRTRTV VTQSQDGGAA CPALVELVAC GDCQVSAWSA FGACDAGTVT
 551  RRRTRTITLQ PIGDGAACPV LEDIAACEPI PCQASDWTVW GPCTASSIRQ
 601  RTRTIVTPAK YGGTDCPPLV DQQNCNPVDC QVSPWGPYEL LGATKCRRRT
 651  VLRDADGGKA CPALVETTTC KKVDCVVGDW GSYSACNPIT KQRSRSRVVV
 701  TSPQDGGAAC PALIESVACA PVNCQVSSWG TWSDCSPITG KRTRRRTVTT
 751  PCAYGGTDCP PLIDEDKCQP VDCKVSDWTL WSGCNPLTRT RSRIRSLITP
 801  ASYGGAACPN LLETQVCVPT DCIVSSWSPF TSCDNSNNGS GKERRFRTVL
 851  QQPDGGAACP PLMEERPAPK VNCVVSDWSD WGVCDPVLFV RVHNRSVITE
 901  AKNGGRCCPA LQGTQPCCPK RVDCKVGAWS DYPTCDPASN TQTRTRNVTQ
 951  AAGPGGRACP DLQQTRSCTP SVDCQVGDWS DWGGCSKSKL VRTRTRQVTV
1001  PRTGKGAKCP ALKDTQSCIP VDCTVSDWGD WSACDSVAGL KTRTRTVLQD
1051  ADGGKKCPSL TQTKTCTTNV DCQVSCWGDY GVCDESVWKR TKTRSILVQP
1101  KGKGKACPSL TQTDACPPRD CKVSGWSQYG ACDANGNRNR TRQIVTDVRG
1151  GGKACPPLQE TGVCSAVNCG VTAWGTWSDC DATSNMQTRS RSIQVQPAYG
1201  GTACPPLTQT RSCQKCIVSD WSDWSICTAD TPTRFRTRVV VQQPPPPSTC
1251  GNSDTDDVAV SLQCPVLRDV QPCDAINCTV TDWGDWGDCN PDTYVKTRNR
1301  TIVQPAVRGG TDCPDLTDTA VCPSVDCVMG DWTDWSPCGL NDLMSSRSRQ
1351  VLVPAAFGGA ECPWQMEGVD CPSIDCVLSD WTDWSICTDD TPTQTRERSI
1401  VQDAVRGGAA CDVLAQSRTC PPVNCELSDW KAWQVCDAKT GTKTRTRDIT
1451  RSPQRNGTAC DALQDIQPCD AVDCIVDPNW GDWSACDNTC GKRYKNRQIL
1501  QNPLYGGVAC AALVMEGPCE PVNCAVSAWS DWSDCDASTG MRTQTRNVTQ
1551  QALYGGLPCP ALVQQKPCDP VCCTVSEWSP WTSCDTDDPQ QHRYRNVTQQ
1601  PLYGGTPCPD LTQDVTCPPI NCAVGLWSDY GLCDASSGLR TRTRSATQVP
1651  RHGGADCPAL NSTIACDPVD CKVSDWSDYG ACDAVTFKKT KTRTVVQQPL
```

Fig. 5.6 Inferred amino acid sequence encoded by the putative *Vsv* gene. The sequence of the putative signal peptide is underlined; the sequence encoded by the λgt11-Vsv cDNA clone is printed in italics.

```
1701  YNGAPCPDLT NDLLCDPIHC AVGPWGAWES CDATSQTKTR RRTITTQPKY
1751  GGCACPALTD SAACDPVNCV MGPWSDWSIC NADGTDTSSF RTRSVVQPAL
1801  YGGSACGATR EEVPCGAVNC VVGNWTGWSS CDASCGKKSR TRQVTQRALY
1851  GGAACPALED LANCDAVDCV VGDWDATWSA CDSNSGLRTL TRPVLQAALY
1901  GGKACPVTIK TKSCDPVDCT VNDWQSWGVC NADTGKKTRV RTVKQGDLYG
1951  GCKCPDLTQD APCDPVPCTV GPFSDWTNCT DADATTSRTR PITQIALYGG
2001  DACPSLTETA ACPPVDCVLD SWGPWSDYDA VTGVRTRTRT VLQPSVRGGS
2051  PCDVTLDSQT APPVNCQMGN WTEFGPCNDV AGVKTRSRPI IVAPKYGGLA
2101  CPDQFQNTTC DAVNCVVSEW SSWGACDSNT GNKTRRRDIL QKDKYGGACC
2151  PTLTDSAPCD RVDCVMNDFG PWTSCDATTG AKTRTRTVQV APLYGGSACP
2201  STTEQGFCNP IDCKVSDWSA FGACNTTSGL KTRTRTVTTQ PLYGGAACLP
2251  LTDSAACDPV NCQVGAYGSW SACSAATLTS TRTRKIIVSP AYGGIDCPCL
2301  TETSPCKMPT PTNCTVTAWS DWTKCSSRSG TRTHTRKVVT AATNGGTACP
2351  ALSETGSCAG LTCTMGAWSS WTTGCDSYGM QTRTRVILDD PYTCSDFCEA
2401  TMDWRACTYS NSTNDYSPPA ASLLLASGEM ALEAQAFDED GDPSHVQALY
2451  V*
```

Fig. 5.6 Inferred amino acid sequence encoded by the putative *Vsv* gene.

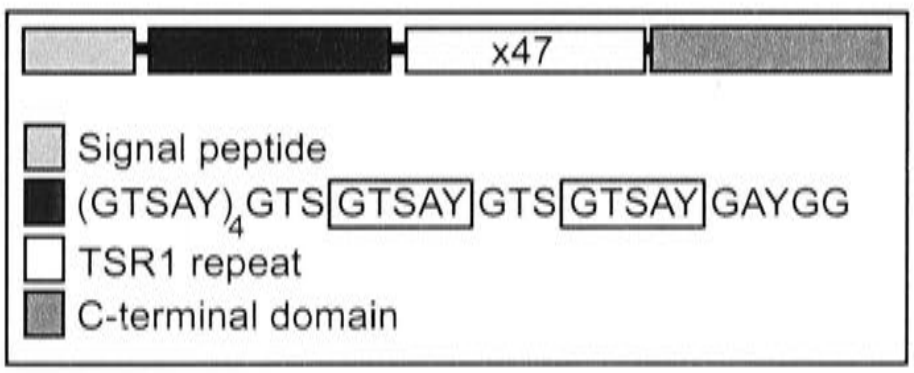

Fig. 5.7 Schematic diagram of the predicted Vsv protein sequence. The N-terminal signal peptide is followed by repeats and variations on the sequence GTSAY, 47 repeats containing a thrombospondin type 1 like motif, and a C-terminal domain.

Vsv protein with TS1 repeats found in proteins of *C. elegans* and *Cryptosporidium parvum*. An alignment of the first seven TS1-like repeats of the Vsv protein is shown in Fig. 5.8B.

Homology to EST sequences obtained from other *Phytophthora* species was not found in the *Phytophthora* database or our laboratory internal database using translated BLAST searches. Searches of the *P. sojae* genome database

with the inferred amino acid sequence of the *Vsv* gene leaving out regions of low complexity detected a single hit along around 300 amino acids with identities of over 75% and similarities over 80%. In addition, matches over the entire length of the inferred protein sequence were found, but the level of similarities was clearly below that of the one mentioned above. Homology to proteins containing TS1 repeats was detected in translated BLAST searches of the non-redundant database at NCBI (see Appendices VII and VIII): at the time, SCO-spondin from cattle showed the highest homologies in BLASTX searches, an F-spondin precursor was the protein with a known function that had highest similarities in TBLASTX searches. Translated searches of the genome of the marine diatome *T. pseudonana* showed no homologues.

```
.DGGWSTWSDWTP.CSASCGFGVQTRDRSCSSPEPKGGQS..CSGLAHQTSL.CDLPA.C
...LVGSWSEWSD.CSTSCGEGNRIRTRE.ITKPPLNGDDSKCPELIEKES..CNKDVEC
...KVGAWSDYPT.CDPA.SNT.QTRTRN.VTQAAGPGGRA.CPDLQQTRS..CTPSVDC
...QVGDWSDWGG.CSKS.KLV.RTRTRQ.VTVPRTGKGAK.CPALKDTQS..CIP.VDC

A

...QVGPWNDYVG.CYSAYSS.TKTRRR.SVIVWPLNNGAA.CPA.LEDTQP.CMPQ.DC
...QVGPWSAYTQ.C.DALCG.KKTRTR.AVILPAQD.GGAACPS.LVDTAP.CDPIN.C
...VVSDWSPWST.C...LLA.KKTRSR.YVQVWSQY.GGTPCPANLLDVQP.CTP.VDC
...AVTDWSPW.....KIIGL.KKTRSR.DITCP.AD.GGKPCPA.LVEEAV.CNP.VDC
...VVSAWS WSGSC.DPTTK.LRTRTR.GIVTAAQD.GGKDCPA.LQETVT.CIDW...
...VVSAWSGFSV.C.NPLTGT.KTRTR.TVVTAAVNN.GADCPT.LVDTTP.CDP.VPC
...VVGNWTSWGS.C.DSKTLQ.RQRTRVVVTTPCY..GGAACPP.LVDTQA.CVPQ.DC
-----------------------------------------------------------
....VS.WS.W...C........KTR.R..V.......GG..CP..L......C.P.V.C

B
```

Fig. 5.8 Alignment of TS1 and TS1-like repeats. The eleven conserved amino acid residues within the TS1 repeats according to Adams and Tucker (2000) are printed in bold. A: TS1 repeats in proteins of *Caenorhabditis elegans* (top row, acc. nr. NM_077715), *Cryptosporidium parvum* (second row, acc. nr. AF017267), and *Phytophthora cinnamomi* (two bottom rows). TS1 repeats of these proteins showed high homology to the predicted Vsv sequence in a translated BLAST search. B: The first seven TS1-like repeats found within the predicted sequence of the Vsv protein; the consensus sequence of the TS1-like repeats in the *P. cinnamomi* Vsv protein is shown underneath the dashed line.

5.3.4. Analysis of genomic DNA from *Phytophthora cinnamomi*, *Phytophthora nicotianae*, and *Phytophthora infestans* using the λgt11-Vsv insert as a probe

Genomic *P. cinnamomi* DNA was digested with the restriction enzymes *Sal*I, *Sac*I, *Eco*RI, and *Xho*I. After gel electrophoresis and blotting, the membrane was probed with the λgt11-Vsv insert. Fig. 5.9 shows the result of the Southern blot. In each digest, one band is strongly labelled. In the *Sac*I and *Xho*I digest, this fragment is approx. 7-8 kbp long, in the case of *Sal*I approx. 3-3.5 kbp, and in the *Eco*RI digest the fragment is approx. 10 kbp long. In the *Eco*RI, *Sac*I, and *Sal*I digests, one more band is weakly labelled, in the *Xho*I digest two. These fragments might represent a second copy or an allele of the recognised gene in *P. cinnamomi*.

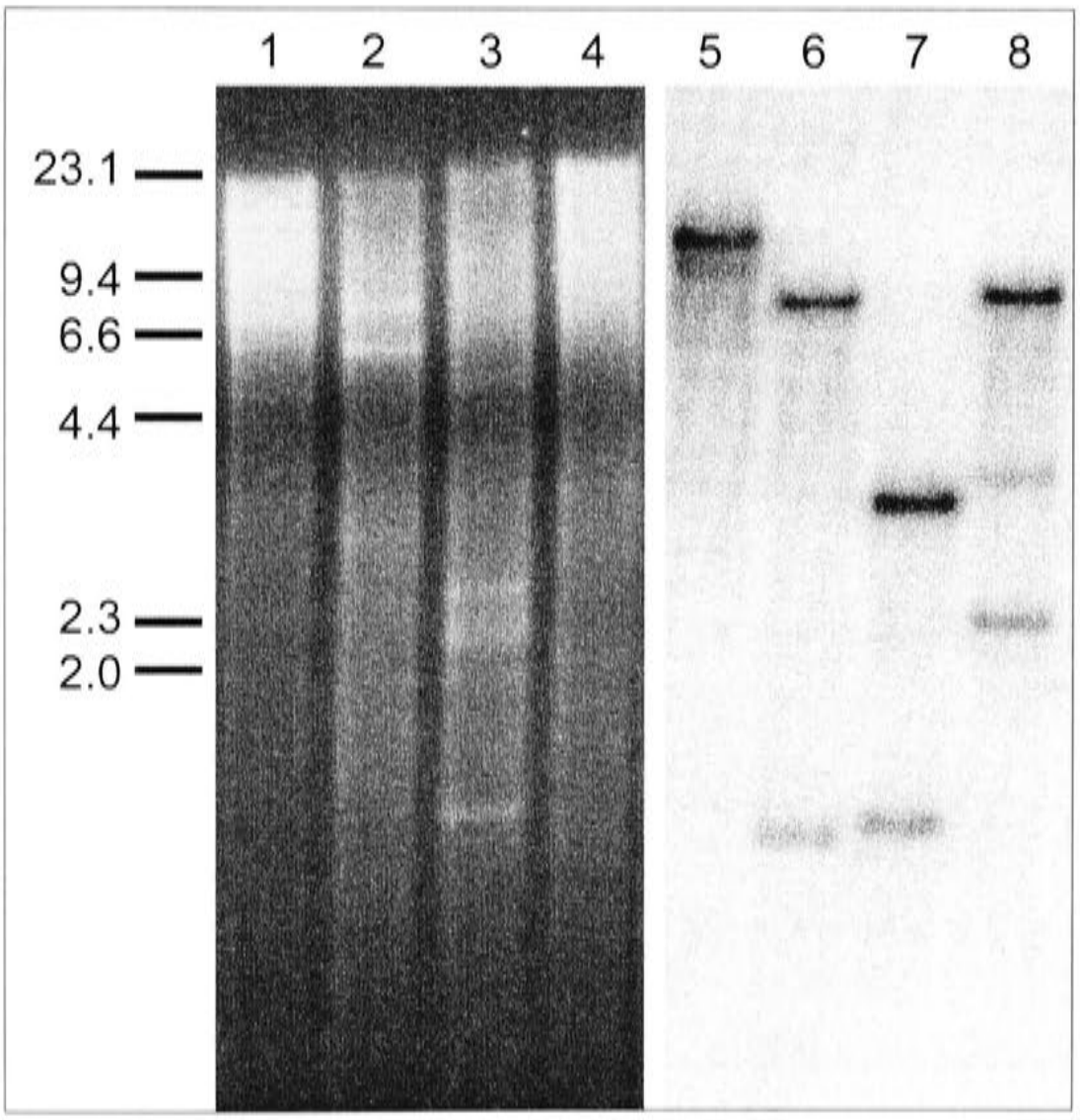

Fig. 5.9 Fragments after restriction digest of genomic DNA from *P. cinnamomi*, gel electrophoresis, and staining with ethidium bromide (lanes 1 – 4). The DNA fragments were blotted onto a membrane and probed with the λgt11-Vsv insert (lanes 5 – 8). The DNA was cut with *Eco*RI (lanes 1 and 5), *Sac*I (lanes 2 and 6), *Sal*I (lanes 3 and 7), and *Xho*I (lanes 4 and 8). Numbers on the left indicate size in kb.

Genomic DNA obtained from *P. cinnamomi*, *P. nicotianae*, and *P. infestans* were digested with *Bam*HI and *Sal*I, separated on an agarose gel, blotted onto a Nylon membrane, and probed with the insert of λgt11-Vsv. Fig. 5.10 shows the results of the Southern blot. In both digests, the probe recognised two DNA fragments in the case of *P. cinnamomi* (compare to Fig. 5.9), and one in the case of *P. nicotianae* and *P. infestans*. The size of the fragments varies from species to species indicating differences in the nucleotide sequence of the putative *Vsv* gene.

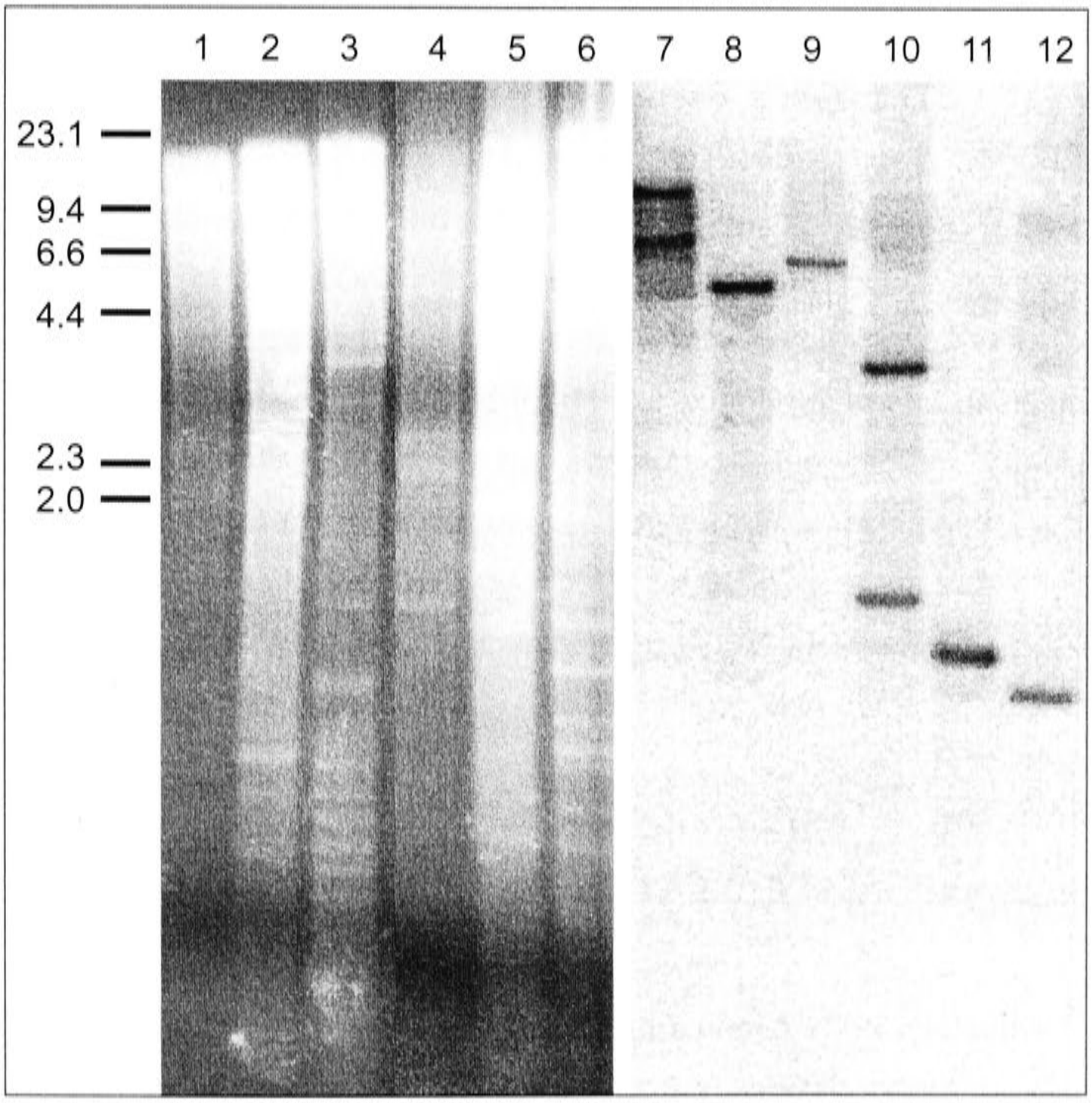

Fig. 5.10 Genomic Southern blot analysis of the Vsv gene in *P. cinnamomi* (lanes 1, 4, 7, and 10), *P. nicotianae* (lanes 2, 5, 8, and 11), and *P. infestans* (lanes 3, 6, 9, and 12). Fragments after restriction digest of genomic DNA, gel electrophoresis, and staining with ethidium bromide (lanes 1 – 6). The DNA fragments were blotted onto a membrane and probed with the λgt11-Vsv insert (lanes 7 – 12). The DNA was cut with *Bam*I (lanes 1 - 3 and 7-9) and *Sal*I (lanes 4-6 and 10-12). Numbers on the left indicate size in kb.

5.4 Discussion

The current chapter reports the cloning and sequencing of the gene encoding the Vsv protein in *P. cinnamomi*. This is the first time a putative spore adhesive of a propagule of an oomycete or true fungal plant pathogen has been characterised on the molecular level. The monoclonal antibodies raised in chapter 4 of this thesis have been successfully used to identify a positive cDNA clone of the appropriate developmental stage. Purification of the clone was followed by DNA sequencing. In a translated search of the non-redundant database the positive cDNA clone showed sequence homology to secreted animal adhesives, the thrombospondin-type 1 repeat (TSR 1) proteins. An open reading frame extended in both directions from the cDNA clone, a genomic clone was isolated and the entire gene sequenced. Comparison of the sequence of the cDNA clone with the genomic clone revealed that four nucleotides differed between the cDNA and genomic clone. Only the difference in position number 1540 would result in a change of amino acids from serine to proline, none would cause an abortion of the protein translation. The differences in nucleotide sequence can be either explained by the fact that during the construction of any cDNA library a reverse transcriptase step is involved which could lead to unwanted base changes or by the poorer quality of sequencing results with lambda clones compared to bacterial plasmids.

5.4.1. Homology of the *Vsv* gene to *Phytophthora sojae* genomic DNA sequences

The identified open reading frame encompassed 7356 nucleotides. When the nucleotide sequence was submitted to the non-redundant or *T. pseudonana* database and nucleotide-nucleotide BLAST searches were carried out, none of the clones in the database showed homology to the proposed *Vsv* gene. The same was true when the *Phytophthora* database was searched, a surprising result considering the reported cDNA with homology to thrombospondin in *P. sojae* by Qutob et al. (2000). It seems the reported sequence is not publicly accessible. In our laboratory internal database, also no homologues were

detected. However, when the *P. sojae* genome database was searched, multiple hits along the entire length of the gene with high rates of identities on the nucleotide level were detected in regions up to 480 nucleotides long. Hence, a homologue to the putative *Vsv* gene seems to be present in *P. sojae* as would be expected from the reported EST sequence in Qutob et al. (2000). An immunological indication for the presence of a *Vsv* homologue in other members of the Oomycetes was the positive result in indirect immunofluorescence assays on zoospores of various members of the genus *Phytophthora* and also in various species of the genera *Pythium*, *Saprolegnia*, *Plasmopara*, and *Albugo* using monoclonal antibody Vsv-1 (Hardham et al. 1991b, Cope et al. 1996, Prof. A.R. Hardham personal communication). The positive result in the *P. sojae* genome search is also in line with the findings in the present study on genomic DNA blots of *P. infestans* and *P. nicotianae* supporting a conservation of the protein at least within the genus *Phytophthora*. In *P. nicotianae* and *P. infestans*, a single band was detected on genomic DNA blots. The occurrence of a second band in *P. cinnamomi* could either be due to the presence of an allele or a second copy of the gene. The position of the more intense band on blots of genomic DNA was identical with the position of the recognised band in DNA blots of the genomic clone that was used to determine the *Vsv* gene sequence.

5.4.2. The *Vsv* gene encodes a thrombospondin type 1 repeat protein with homology in other *Phytophthora* species

The inferred amino acid sequence is 2452 residues long and a molecular weight of 261.6 kDa and a pI of 5.52 were predicted with the software protparam. In *P. cinnamomi*, the predicted molecular weight is approx. 40-50 kDa above the apparent molecular weight on SDS-PAGEs reported in chapter 4 of the present study. In previously published studies the apparent molecular weight was slightly above a 220 kDa marker band (Hardham and Gubler 1990). The difference in predicted versus apparent molecular weight could be due to various reasons. Firstly, the gene could contain an intron. On the one hand, the software FGENESH set at *C. elegans* as the model organism identified a

single exon of 7356 nucleotides, but on the other hand, the gene was predicted to terminate 65 nucleotides earlier when the protist *Plasmodium falciparum* was selected as a model (not shown). It is also possible that in *P. cinnamomi* non-canonical splice sites are recognised. These possibilities will have to be addressed in future RNA studies. Secondly, it is difficult to estimate the molecular weight of high molecular weight proteins since the molecular weight is calculated from their position on a gel using marker bands to generate a standard curve. This means that small differences in the position of high molecular weight proteins on the gel lead to significant differences in their calculated apparent molecular weight. Thirdly, it is well known that proteins often do not migrate according to their molecular weight. The latter reason is the most likely, considering the many reports in the literature that usually describe errors of 10-30% when the molecular weight is estimated from protein gels (e.g. Chait and Kent 1992). For example, the predicted molecular weight of thrombospondin-1 monomers is 127.5 kDa, whereas the molecular weight was estimated on SDS-PAGEs to be 185 kDa (Lawler and Hynes 1986, Lawler et al. 1982); the difference in that case is 45%.

Translated searches of the *P. sojae* genome database identified a single hit along 300 amino acids with identities of over 75% and similarities of over 80% as well as other, less homologous sequences. However, some homologies were still significant, giving additional support to the idea of the presence of a true homologue in *P. sojae*. In searches of the non-redundant database with the Vsv amino acid sequence homologues belonging to the TSR 1 protein superfamily were detected. Analysis of the inferred amino acid sequence of the putative *Vsv* gene revealed differences and similarities to members of this quickly growing protein family (e.g. TRAP-C1 in *Cryptosporidium parvum* [apicomplexa], Spano et al. 1998; EtMIC4 in *Eimeria tenella* [apicomplexa], Tomley et al. 2001; ADAMTS-1 in *Mus musulus* [mammalia], Kuno et al. 1997, PbTRAP in *Plasmodium berhei* [apicomplexa], Robson et al. 1997; thrombospondin from *Haemonchus contortus* [nematode], Skuce et al. 2001, EmTFP250 in *Eimeria maxima* [apicomplexa], Witcombe et al. 2003). The TSR 1 protein superfamily includes the TSR 1-like proteins found in apicomplexan organisms and will be referred to as TSR 1 proteins in the following discussion. TSR 1 proteins are characterised by the TS1 repeats.

These contain a number of conserved amino acid residues. The current study used the consensus sequence established by Adams and Tucker (2000) for comparison with the inferred amino acid sequence of the Vsv protein.

A promising indication that the *P. cinnamomi* adhesive could be a TSR 1 protein is the fact that these (glyco-)proteins function as secreted adhesives serving in a variety of pathways (e.g. reviewed for thrombospondin-1 and -2 by Adams and Tucker 2000). For example, neuronal development of mammals is influenced by F-spondin (Tzarfati-Majar et al. 2001). SCO-spondin, a high molecular weight TSR 1 glycoprotein secreted during early neuronal development by the subcommissural organ of mammals was first cloned and sequenced in cattle (Meiniel et al. 1995, Nualart et al. 1998, Didier et al. 2000). Homologues of SCO-spondin can be detected on genomic DNA blots of various members of the chordate phylum (Gobron et al. 1999). The same study showed immunological evidence of the localisation of immunoreactive glycoproteins to the subcommissural organ of higher chordates or the cerebral vesicle of lower chordates indicating the importance and phylogenetic conservation of the molecule. Another important function of TSR 1 proteins is their involvement in angiogenesis in mammals. For example, some members of the ADAMTS-subfamily show angioinhibitory properties (ADAMTS-1, Iruela-Arispe et al. 2003). One study addressed the direct involvement of the TS1 repeats in thrombospondin-1 and found that they inhibited neovascularisation and were crucial in the suppression of tumour growth *in-vitro* (Miao et al. 2001).

The Vsv protein as well as other TSR 1 proteins shows a modular make-up (e.g. reviews by Adams and Tucker 2000 or Tomley and Soldati 2001). The Vsv protein consists only of a leader sequence, a region of low complexity containing a number of repeats and variations on the GTSAY motif which has no matches in the non-redundant database, 47 TS1 repeats, and a C-terminal domain. Other TSR 1 proteins have additional 'modules' or domains that differ from the TS1 repeats, such as von Willebrand domains, A-type domains, thrombospondin-type 2 or 3 repeats, epidermal growth factor-like calcium binding domains, lagrin domains or Kunitz inhibitor domains (e.g. Adams and Tucker 2000, Witcombe et al. 2003, Skuce et al. 2001, Goncalves-Medes et al. 2003, Trottein et al. 1995, Robson et al.1997, Didier et al. 2000). TSR 1

proteins include proteins with a wide range of sizes and another similarity that is exhibited by some members of TSR 1 proteins with the Vsv protein is their large size. Currently, the largest known TSR 1 protein is SCO-spondin of cattle with a transcript size of 14 kbp (Didier et al. 2000).

Adams and Tucker (2000) reported the 11 amino acid residues W8, S9, W11, C14, C18, R25, R27, C29, C41, C51, and C56 as conserved in the TS1 repeats. The TS1-like repeats of the Vsv protein lack two cysteine residues that are present in the consensus sequence of TS1 repeats of animals (C18 and C29), probably affecting the three dimensional structure of the proteins. However, as shown in the alignment in Fig. 5.8B not all TS1 repeats in animal proteins contain all six cysteine residues that are conserved in others and it seems that the two cysteine residues that are missing in *P. cinnamomi* are less conserved than the other four. The other nine amino acid residues of the TS1 repeat consensus are present in the 47 TS1-like repeats of the *P. cinnamomi* Vsv protein. However, twelve additional residues appear in 22 or more TS1-like repeats of the Vsv protein (compare Fig. 5.8A).

A difference of most TSR 1 proteins to the Vsv protein seems to be their glycosylation to varying degrees, for example, SCO-spondin or thrombospondin-1 (e.g. reviewed in Meiniel 2001, Lawler and Hynes 1986). This finding is in accordance with that on many other types of adhesives of, for example, fungi and animals: a glycoprotein has been implicated in the adhesion of encysting zoospores of *Py. aphanidermatum* or in the adhesion of *Nectria haematococca* conidia (Estrada-Garcia et al. 1990a, Kwon and Epstein 1993). The Vsv protein on the other hand does not seem to contain a carbohydrate moiety. On protein gels or immunoblots, the Vsv protein always appears as a sharp band. Glycoproteins can give rise to some degree of smearing in these assays (e.g. Pain et al. 1994, Kwon and Epstein 1997). However, the latter is not true for the Lpv proteins in *P. cinnamomi*. The epitope of monoclonal antibody Lpv-1 indicates a glycoprotein, but the Lpv proteins often give clear bands in SDS-PAGEs and immunoblots (Gubler and Hardham 1990 or this study, chapter 1). The epitopes of all monoclonal antibodies towards the Vsv protein in *P. cinnamomi* or *P. nicotianae* have a proteinaceous nature (Hardham and Gubler 1990; this study, chapter 4). In many cases where monoclonal

antibodies are raised towards a glycoprotein, the carbohydrate epitope dominates the immune response of the animal (e.g. Cpa antigen in *P. cinnamomi*, Hardham et al. 1986). If the Vsv protein were glycosylated, at least some of the monoclonal antibodies would be expected to recognise a carbohydrate epitope. However, the software programs NetNGlyc (http://www.cbs.dtu.dk/services/ NetNGlyc) and NetOGlyc (http://www.cbs.dtu.dk/services/NetOGlyc) that are designed for predicting N- and O-glycosylation sites in human proteins, respectively, have found potential glycosylation sites at some amino acid residues of the predicted Vsv protein sequence (not shown). Deglycosylation experiments could be used to shed some light onto this matter. However, if the position of the band labelled by monoclonal antibody Vsv-1 did not shift it would not necessarily prove absence of glycosyslation since only the removal of large amounts of carbohydrate would give significant changes in the apparent molecular weight of the Vsv protein whereas in the case of marginal glycosylation their cleavage could remain undetectable, a complication that arises from the large size of the Vsv protein.

5.4.3. Expression of the *Vsv* gene – preliminary data

The Vsv protein is implicated in the adhesion of zoospores. As mentioned in chapter 1 it is produced once sporulation is induced in the mycelium and stored in small vesicles within the zoospores until its release upon encystment. In order to find out more about the expression of the *Vsv* gene, I carried out preliminary RNA analysis (not described). An RNA dot blot with hyphal samples collected between 0 and 24 h after induction of sporulation was incubated with the insert of cDNA clone λgt11-Vsv and showed a weak signal present between 2 to 8 h after the induction of sporulation. However, several attempts to show expression of the *Vsv* gene on an RNA blot after gel electrophoretic separation of all RNA species of hyphal material collected 4 h after the induction of sporulation failed so far. It is possible that the mRNA for the Vsv protein is an unstable species. To address this possibility in future work real-time PCR or in situ hybridisation could be tried out.

5.4.4. Concluding remarks

Summarising the results in this chapter of my thesis I have gained knowledge about the molecular bases of *P. cinnamomi* zoospore adhesion, one of the fundamental processes involved in pathogenicity. It is the first report on the molecular cloning and sequencing of a spore adhesive of a fungal plant pathogen. The finding of a molecule with homologues in other parasitic protists, the apicomplexans, is exciting indeed. In the apicomplexans, TS1 repeats seem to play a crucial role in the infection of host cells (Matuschewski et al. 2002). Using immunolabelling it has been shown that the TSR 1 protein MIC2 present in the micronemes of *Toxoplasma gondii* is released upon binding to a host cell (Carruthers and Sibley 1997).

The finding of TSR 1 proteins in *P. cinnamomi* has also interesting phylogenetic implications. The TSR 1 proteins seem to have evolved before animals and protists separated, yet this superfamily does not have homologues in plants or true fungi: The complete genome sequences for *Arabidospis thaliana*, *Saccharomyces cerevisiae*, and *Neurospora crassa* are available, yet no TSR 1 proteins have been registered for these organisms in the non-redundant database. It seems that the TSR 1 proteins have evolved before the separation of eukaryotes into various kingdoms and have been lost in plants and true fungi; their function could have been taken over by other types of adhesives.

Chapter 6 Does the *Vsv* gene code for the Vsv protein?

6.1 Introduction

In the previous chapter the cloning and sequencing of the putative *Vsv* gene was described: Immunological screening of a cDNA expression library yielded a positive clone that encoded part of the putative *Vsv* gene. It was used to purify a genomic clone and obtain the entire gene sequence. When using an immunological cloning strategy it is important to test that the sequenced gene indeed encodes the protein in question. In order to do so several approaches can be taken. An immunological approach, peptide mass fingerprinting or protein sequencing can be carried out. In the present study, all these approaches were attempted.

6.1.1. Immunological methods

Antibodies recognise small parts of proteins specifically; these parts are termed epitopes (see chapter 4). In the current study, a mixture of monoclonal antibodies Pn-Vsv-1, Pn-Vsv-2, Pn-Vsv-3, and Pn-Vsv-4 was used to purify a cDNA clone, λgt11-Vsv, encoding part of the putative Vsv protein. In order to show that a certain cDNA clone indeed codes for the protein in question, polyclonal antibodies can be made monospecific: the fusion protein encoded by the cDNA clone is expressed and used to purify anti-fusion protein antibodies from the polyclonal antibodies. These monospecific polyclonal antibodies are then used in immunological assays like immunofluorescence or immunogold assays to show, for example, the subcellular localisation of their antigen (Harlow and Lane 1999). For example, Marshall et al. (2001) used this strategy successfully to show that their cDNAs encoding parts of the putative Lpv protein were indeed recognised by monospecific anti-Lpv cDNA polyclonal antibodies.

6.1.2. Peptide mass fingerprinting

Mass spectrometry (MS) has become an important tool in proteome research but also for peptide mass fingerprinting of individual proteins. In MS, either whole proteins or protein fragments resulting from enzymatic or chemical treatment are subjected to ionisation; a mass analyser and a detector are coupled to the ionisation chamber under vacuum (e.g. Corthals et al. 2000). The mass spectrometer gives mass *m* over charge *z* ratios of the ionised proteins or protein fragments. Data are displayed in the form of graphs or 'spectra' with the *m*/*z* ratio on the x-axis and the ion intensity on the y-axis. Initially, the identification of large molecules was hampered by decomposition of the molecules during the ionisation. However, the development of two 'soft' ionisation technologies has been important for medical and biological research since their invention in the late 1980s: electrospray ionisation (ESI; Fenn et al. 1989) and matrix-assisted laser desorption/ionisation (MALDI; Karas and Hillenkamp 1988). Usually, ESI mass spectrometers are coupled with a (quadrupole) ion trap (IT) as a mass analyser, and MALDI mass spectrometers with a time-of-flight (TOF) mass analyser.

In ESI MS, peptides are ionised by spraying the sample from the tip (1-5 µm) of a capillary at high voltages. The fine droplets evaporate and the ionised peptides end up in the vacuum of the IT of the MS. The IT is generated by a radio frequency trapping field. Resonance ejection of unstable ions results when the radio frequency voltage is increased and at the same time voltage on the two end caps of the IT is applied. With increasing radio frequency voltage ions of increasing *m*/*z* ratios become unstable, are ejected from the IT, and detected.

In MALDI MS, the sample is mixed with a matrix compound such as 3,5-dimethoxy-4-hydrocinnamic acid (sinapinic acid). The sample/matrix mixture is allowed to crystallise onto the probe. Ionisation is achieved by irradiating the sample/matrix crystals with a laser beam *in-vacuo* while applying a high voltage. The photons from the laser beam are absorbed by the sample/matrix and this energy is dissipated as heat. The resulting rapid increase in temperature is followed by desorption of the sample/matrix crystals into the gas phase. This sublimation event is characterised by the sudden expansion of the crystal

causing ionisation of the sample/matrix molecules. Since the sample/matrix is under high voltage, ionised molecules are repelled and accelerated in the TOF mass analyser. In theory, all ions are accelerated at the same rate, so the 'flight-time' they need to reach the detector is dependent only on their mass: small/light ions reach the detector faster than large/heavy ones. Typically, slight variation in the kinetic energy of otherwise identical ions is observed, limiting the resolving power of the linear MALDI-TOF instruments just described. In order to overcome this problem, for example, ion mirrors can be used (see reviews by Corthals et al. 2000, Braun and Neusser 2002).

ESI and MALDI MS are fast developing technologies and some of the important technical improvements in MALDI MS are mentioned in the following paragraphs.

Initially, only UV lasers (e.g. 337 nm from a nitrogen laser) were used to ionise the sample in MALDI-TOF (see reviews by Chait and Kent 1992 or Corthals et al. 2000). Now, also IR lasers are available (see Gross and Strupat 1998).

Often, MALDI MS is employed after 2D-gel electrophoresis. Although both technologies have limitations, they have facilitated the characterisation of the proteome of tissues, cells or subcellular compartments (e.g. the plasma membrane proteins of *Arabidopsis thaliana*: Santoni et al. 1998 and 1999). The automation of sample handling after 2D-gel electrophoresis has received much attention. For example, Nordhoff et al (2001) described automated sample processing for MALDI-TOF MS. An important development in the preparation of samples for MALDI MS after 2D-gel electrophoresis is described in Stensballe and Jensen (2001). The authors developed a procedure which employs in-gel reduction and S-carbamidomethylation of proteins in intact silver-stained gels, the transfer of protein-containing gel pieces onto the probe, and subsequent on-probe tryptic digest and sample co-crystallisation with the matrix material. This procedure reduces the number of pipetting steps and also contamination of the sample with human keratin, a common problem in MS after gel electrophoresis. After MS, data analysis and database search has to be carried out. Again, recent developments facilitate faster and more reliable analysis (for recent proteome analysis and their strategies see review by Liska and Shevchenko 2003).

The application of MS seems without boundaries and more and more effort is made to employ MS data to analyse, for example, the tertiary structure of proteins (e.g. disulfide bonds in proteins: review by Gorman et al. 2002), the presence of posttranslational modifications (e.g. with the software FindMod after MS analysis, Wilkins et al. 1999), oligonucleotides (see reviews by Koomen et al. 2002 or Tang et al. 2003), mixtures of proteins to identify bacterial strains (reviewed in Lay 2001) or even the subcellular localisation of certain proteins within tissue sections (reviewed in Chaurand and Caprioli 2002).

6.1.3. Protein sequencing

If peptide mass fingerprinting does not give satisfactory results, protein sequencing can be employed. Protein sequencing is more tedious and expensive than peptide mass fingerprinting. Certain MS methods can be used to obtain internal sequence information (e.g. ESI MS/MS, see review by Corthals et al. 2000). For terminal sequencing chemical methods are available. The easiest way to get sequence information using chemical protein sequencing is by Edman degradation (e.g. reviewed by Lottspeich 2000). Sequence information is obtained from the N-terminus of the protein. Phenylisothiocyanate reacts with the free amino group of the N-terminal residue to give a phenylisothiocarbamylpeptide. After this coupling reaction the residue is cleaved off the protein and converted into a cyclic molecule, a phenylthiohydantoin derivative (also called anilinothiazolinone amino acid derivative) that can be identified. The number of residues that can be obtained depends on the efficacy with which the residues are cleaved off from the protein (reviewed in Lottspeich 2000).

C-terminal sequencing is an option, but it is much harder to obtain sequence information this way. Amongst other methods, enzymatic reactions are employed to cleave off C-terminal residues. The disadvantages of this strategy include endopeptidase contamination of carboxypeptidases and non-linear reaction kinetics of the enzymes, the latter resulting in complex and difficult-to-interpret data (e.g. Yarwood 1989 or Tsugita 1987).

6.2 Materials and Methods

6.2.1. Monospecific polyclonal antibodies directed towards the fusion protein encoded by cDNA clone λgt11-Vsv

λgt11-Vsv was plated on *E. coli* Y1090 host cells on LB agar and overlayed with nitrocellulose circles wetted with IPTG after 4 h growth at 37°C in the dark. These membranes are referred to as Vsv-membranes. The membranes were incubated overnight at 37°C in the dark. In order to avoid binding of non-specific antibodies to the Vsv-membrane a pre-clearing step was carried out. A second membrane circle (pre-clearing membrane) was incubated for 90 min in TBST containing 5% skim milk powder, rinsed three times for 5 min with TBST, and added to 10 mL purified Immunoglobulin G polyclonal anti-*P. cinnamomi* zoospore antibody solution (Marshall et al. 2001) diluted to 400 µg mL^{-1} in TBST. It was incubated for 45 min on a horizontal shaker and the membrane removed. In the meantime, the Vsv-membrane had been prepared: after a 90 min blocking step in TBST containing 5% skim milk powder it had been rinsed three times for 5 min in TBST. The Vsv-membrane was incubated in the pre-cleared polyclonal antibodies overnight at RT. It was washed with TBST as before and the monospecific polyclonal antibodies were eluted into 4 mL 0.1 M glycine-HCl pH 2.7 over a period of 10 min. The membrane was removed and the antibody solution neutralised with 300 µL 1 M Tris-HCl pH 9.1. The monospecific polyclonal antibodies were concentrated in an Ultrafree® 15 centrifugal filter device (Biomax 50 K NMWL membrane, Millipore) to a final volume of 300 µL and used in indirect immunofluorescence assays on *P. cinnamomi* zoospores as described in section 2.2.6.

6.2.2. Matrix Assisted Laser Desorption/Ionisation Time of Flight Mass Spectrometry and peptide database searches

After separation of a crude *P. cinnamomi* zoospore protein sample on a 6% SDS-PAGE (as detailed for *P. nicotianae* zoospore proteins in section 4.2.5), the Coomassie Brilliant Blue stained band that corresponded to the one labelled

by the Vsv antibodies (see section 4.3.6) was cut out and stored at RT until a 16 h in-gel tryptic digest at 37°C was carried out at the Australian Proteome Analysis Facility in Sydney. The resulting peptides were extracted from the gel with 50% (v/v) acetonitrile containing 1% (v/v) trifluoroacetic acid (TFA) solution. A 1 µL aliquot was spotted onto a sample plate with 1 µL of matrix (α-cyano-4-hydroxycinnamic acid, 8 mg mL^{-1} in 50% v/v acetonitrile, 1% v/v TFA) and allowed to air dry. MALDI-TOF MS was performed with a Micromass TofSpec 2E Time of Flight Mass Spectrometer, a nitrogen laser (337 nm) was used to irradiate the sample. The spectra were acquired in reflectron mode in the mass range 600 to 3500 Da. A near point calibration was applied giving a typical mass accuracy of approx. 100 ppm or less. The monoisotopic peak list of the peptides from the sample was compared to one obtained in a theoretical digest of the Vsv protein. The theoretical digest was performed using the PeptideMass software on the ExPasy server (Wilkins et al. 1997 and Wilkins et al. 1998; http://kr.expasy.org/tools/peptide-mass.html). The NCBI database was searched with the actual MALDI-TOF MS peptide masses that matched the predicted Vsv fragment masses using the software ProFound (Zhang and Chait 1995, Zhang and Chait 1998, and Zhang and Chait 2000; http://prowl.rockefeler.edu/cgi-bin/ProFound). The software program Peptide Search/WWW (http://www.mann.embl-heidelberg.de/GroupPages/ PageLink/ peptidesearch/ Services/) was also used to search for any proteins with matching tryptic peptide fragment masses. In both cases, the settings were as follows: taxonomic category: eukaryotes, approx. protein mass: 0-400 kDa, digest chemistry: trypsin, cysteine residues not modified, maximum number of missed cleavage sites: 1, monoisotopic peptide masses with 0.1 Da mass tolerances, and peptide charge state: protonated.

6.2.3. N-terminal sequencing of the putative Vsv protein

After separation of a crude *P. cinnamomi* zoospore protein sample on a 6% SDS-PAGE and overnight blotting of the proteins onto a PVDF membrane, an immunoblot was carried out as described in section 4.2.5. Monoclonal antibody Pn-Vsv-4 was used as a primary antibody and TBST served as a negative control. The remainder of the blot was stained with Coomassie Brilliant Blue.

The Coomassie stained band that corresponded to the one labelled by the Vsv antibody on the immunoblots was cut out and sent to the Biomolecular Recource Facility at the John Curtin School of Medical Research at the Australian National University for N-terminal sequencing. Twelfe cycles were carried out on approx. 0.5 pMol of protein on an Applied Biosystems Procise Sequencer following standard methods.

6.2.4. Towards purification of the Vsv protein

At first, immunopurification using membrane-adsorbed primary antibody Pn-Vsv-3 as described in section 2.2.7 was attempted. After moderate success, partial purification using ammonium sulphate (AS) precipitation was attempted. 51.5 g of AS were dissolved in 100 mL distilled water at RT to give a 3.9 M solution and the pH adjusted with 10 N NaOH to 7.0-7.5 (referred to as 100% AS solution). Freeze dried zoospores were dissolved in 8 M urea and 100% AS solution added to give a final concentration that corresponded to 20% of the 100% AS solution. After incubation for 5-10 min at 4°C on a rotary shaker the precipitate was removed by centrifugation at 14000 x g for 15 min at 4°C. The supernatant was used for subsequent steps (40%, 60%, 80% AS precipitation) that were carried out as for the 20% AS precipitation. The pellets were dissolved in 8 M urea, precipitated twice with a mixture of tributylphosphate:acetone:methanol (1:12:1) by adding twice the amount of mixture to the sample and incubation overnight at 4°C, centrifuged for 15 min at 14000 x g, finally taken up in 75 µL 8 M urea, and subjected to SDS-PAGE. The gels were stained in Coomassie Brilliant Blue as described in section 2.2.9.

6.3 Results

6.3.1. Monospecific polyclonal antibodies towards the β-galactosidase fusion protein encoded by λgt11-Vsv

In order to verify the authenticity of the cDNA clone purified with help of the monoclonal antibodies Pn-Vsv-1, Pn-Vsv-2, Pn-Vsv-3, and Pn-Vsv-4, λgt11-Vsv, the fusion protein encoded by the cDNA clone was used to affinity purify polyclonal antibodies. The monospecific polyclonal antibodies and the initial polyclonal serum were used in indirect immunofluorescence assays. In these assays the monospecific polyclonal antibodies labelled the large peripheral vesicles but not the ventral vesicles specifically (data not shown). This result is not in accordance with the expectations. The initial polyclonal serum (positive control) labelled entire zoospores brightly.

6.3.2. Peptide mass fingerprinting and database searches

A Coomassie Brilliant Blue stained band corresponding to the one recognised by the Vsv antibodies was subjected to tryptic digest and peptide mass fingerprinting. Fig. 6.1 shows the results obtained in the peptide mass fingerprinting. The peaks obtained in the fingerprinting were compared to the monoisotopic peaks obtained in a theoretical digest of the putative Vsv protein. One peak that was expected in a theoretical digest was identified in the actual experiment (1587.9217 Da; Table 6.1), another two masses could result from fragments arising after incomplete tryptic digestion: one missed cleavage was permitted in the theoretical digest (801.4689 and 1805.7979 Da; Table 6.2). Database searches using ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) or PeptideSearch/WWW (http://www.mann.embl-heidelberg.de/GroupPages/PageLink/peptidesearch /Services) were performed with the masses of the three positive fragments. Both programs identified various proteins with matching tryptic peptide fragment masses (not shown).

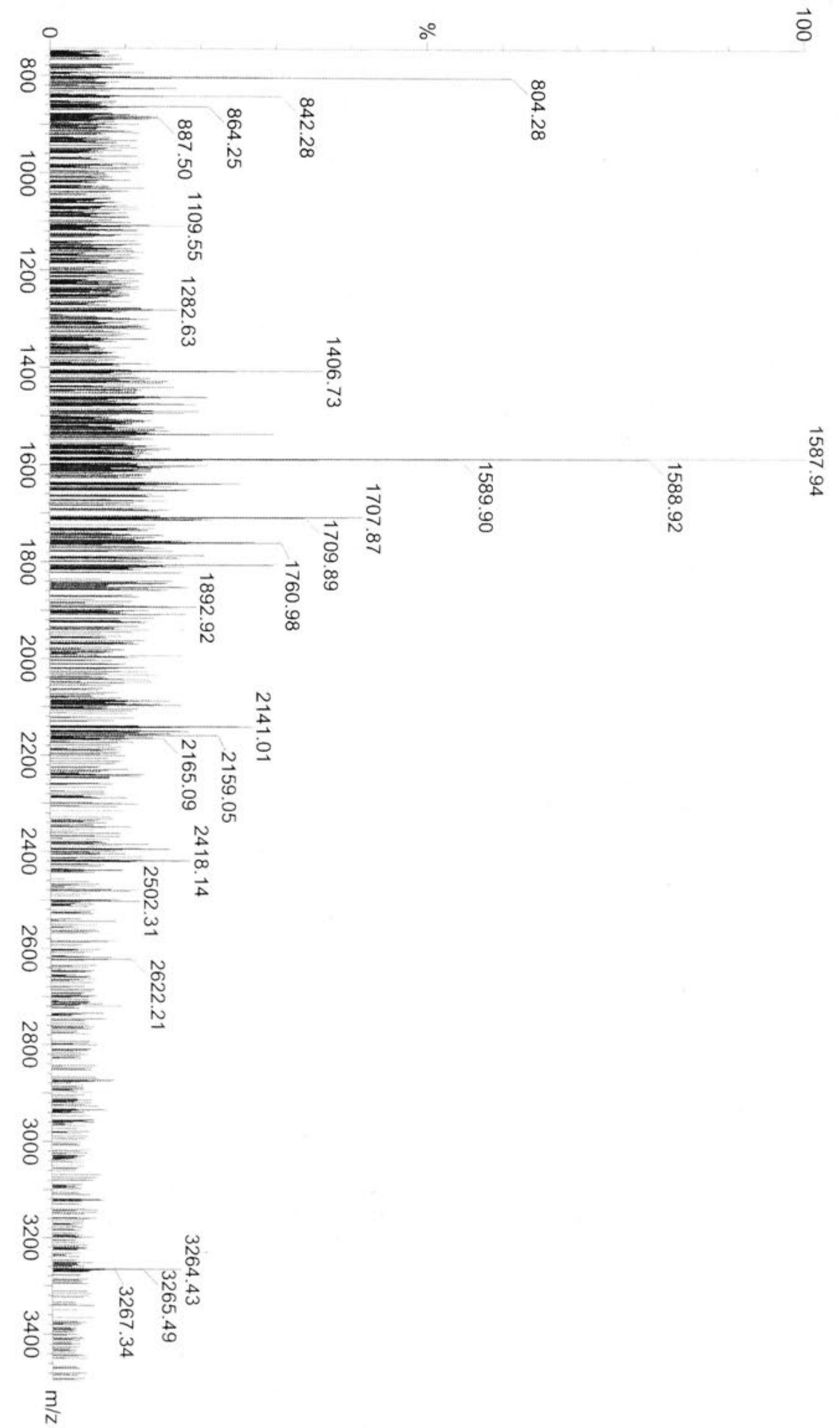

Fig. 6.1 MALDI-TOF MS spectrum acquired in reflectron mode after tryptic digest of a protein band containing the Vsv protein and obtained by 1D SDS-PAGE. The *m/z* values correspond to the molecular weight of the detected fragment in Da.

Position	Mass	Peptide sequence
292-302	1016.5371	GIVTAAQDGGK
303-334	3427.6007	DCPALQETVTCIDWVVSAWS GFSVCNPLTGTK
440-466	2862.3106	TVIQACDGGAACPPLQEEVQ YQPVDCK
470-486	1785.8125	WVWDANGAGACPPGTQR
642-653	1236.5962	ACPALVETTTCK
655-673	2013.9044	VDCVVGDWGSYSACNPITK
729-749	2195.9835	TVTTPCAYGGTDCPPLIDED K
750-756	792.3379	CQPVDCK
757-771	1734.8268	VSDWTLWSGCNPLTR
830-852	2405.1951	TVLQQPDGGAACPPLMEERP APK
853-873	2395.1209	VNCVVSDWSDWGVCDPVLFV R
889-902	1448.6153	CCPALQGTQPCCPK
908-926	2068.9029	VGAWSDYPTCDPASNTQTR
929-939	1027.5279	NVTQAAGPGGR
940-948	1031.4938	ACPDLQQTR
949-969	2216.8681	SCTPSVDCQVGDWSDWGGCS K
996-1023	2958.2590	DTQSCIPVDCTVSDWGDWSA CDSVAGLK
1028-1037	1003.5054	TVLQDADGGK
1039-1046	877.4448	CPSLTQTK
1047-1071	2798.1201	TCTTNVDCQVSCWGDYGVCD ESVWK
1077-1083	784.4927	SILVQPK
1088-1101	1459.6668	ACPSLTQTDACPPR
1105-1120	1684.7132	VSGWSQYGACDANGNR
1125-1131	830.4730	QIVTDVR
1174-1193	2088.0542	SIQVQPAYGGTACPPLTQTR
1198-1216	2155.9423	CIVSDWSDWSICTADTPTR
1221-1250	3121.5292	VVVQQPPPPSTCGNSDTDDV AVSLQCPVLR
1251-1278	3130.2863	DVQPCDAINCTVTDWGDWGD CNPDTYVK
1283-1290	883.5359	TIVQPAVR
1381-1388	887.4945	SIVQDAVR
1389-1400	1147.5524	GGAACDVLAQSR
1401-1413	1491.6606	TCPPVNCELSDWK
1414-1421	920.4294	AWQVCDAK
1627-1633	758.4155	SATQVPR
1634-1654	2086.8990	HGGADCPALNSTIACDPVDC K
1655-1670	1763.7581	VSDWSDYGACDAVTFK
1725-1731	788.4512	TITTQPK
1776-1792	1636.8111	SVVQPALYGGSACGATR
1793-1819	2758.1364	EEVPCGAVNCVVGNWTGWSS CDASCGK
1871-1885	1587.9217	TLTRPVLQAALYGGK
1895-1918	2600.0486	SCDPVDCTVNDWQSWGVCNA DTGK
1927-1935	940.4193	QGDLYGGCK
2022-2029	899.5309	TVLQPSVR
2069-2077	980.6251	SRPIIVAPK
2127-2143	1729.6978	YGGACCPTLTDSAPCDR
2144-2164	2217.9249	VDCVMNDFGPWTSCDATTGA K
2169-2196	2886.3106	TVQVAPLYGGSACPSTTEQG FCNPIDCK
2197-2213	1743.8006	VSDWSAFGACNTTSGLK
2268-2289	2267.0756	IIVSPAYGGIDCPCLTETSP CK
2290-2306	1938.8724	MPTPTNCTVTAWSDWTK
2368-2387	2379.9930	VILDDPYTCSDFCEATMDWR

Table 6.1 Theoretical tryptic digest of the predicted Vsv protein using the PeptideMass software (http://kr.expasy.org/tools/peptide-mass.html). In this theoretical digest no cleavage sites were missed. Fragments between 750 and 3500 Da are shown. The monoisotopic masses giving peptide masses as [M+H+] are displayed. All cysteins were set to be not modified, the methionines were set as not oxidised. The protein fragment that has been confirmed using MALDI-TOF MS is shaded.

Position	Mass	Peptide sequence
290-302	1273.6858	TRGIVTAAQDGGK
380-385	801.4689	TLQRQR
428-435	966.5003	NTDGLRYK
438-466	3105.4438	SRTVIQACDGGAACPPLQEE VQYQPVDCK
440-469	3176.5060	TVIQACDGGAACPPLQEEVQ YQPVDCKVSK
467-486	2100.0079	VSKWVWDANGAGACPPGTQR
470-488	2042.9613	WVWDANGAGACPPGTQRTR
584-592	986.5993	TRTIVTPAK
632-641	1031.5480	TVLRDADGGK
636-653	1779.8251	DADGGKACPALVETTTCK
642-654	1364.6912	ACPALVETTTCKK
654-673	2141.9994	KVDCVVGDWGSYSACNPITK
655-675	2298.0641	VDCVVGDWGSYSACNPITKQ R
728-749	2352.0846	RTVTTPCAYGGTDCPPLIDE DK
729-756	2969.3035	TVTTPCAYGGTDCPPLIDED KCQPVDCK
750-771	2508.1468	CQPVDCKVSDWTLWSGCNPL TR
757-773	1991.9756	VSDWTLWSGCNPLTRTR
828-852	2708.3646	FRTVLQQPDGGAACPPLMEE RPAPK
853-877	2901.3923	VNCVVSDWSDWGVCDPVLFV RVHNR
874-884	1253.6960	VHNRSVITEAK
878-888	1131.6116	SVITEAKNGGR
885-902	1832.8023	NGGRCCPALQGTQPCCPK
889-903	1604.7164	CCPALQGTQPCCPKR
904-926	2514.1024	VDCKVGAWSDYPTCDPASNT QTR
908-928	2326.0517	VGAWSDYPTCDPASNTQTRT R
927-939	1284.6767	TRNVTQAAGPGGR
929-948	2040.0039	NVTQAAGPGGRACPDLQQTR
940-969	3229.3441	ACPDLQQTRSCTPSVDCQVG DWSDWGGCSK
949-971	2431.9951	SCTPSVDCQVGDWSDWGGCS KSK
977-984	956.5636	TRQVTVPR
979-987	985.5789	QVTVPRTGK
988-995	787.4494	GAKCPALK
991-1023	3470.5371	CPALKDTQSCIPVDCTVSDW GDWSACDSVAGLK
996-1025	3215.4078	DTQSCIPVDCTVSDWGDWSA CDSVAGLKTR
1026-1037	1260.6542	TRTVLQDADGGK
1028-1038	1131.6004	TVLQDADGGKK
1038-1046	1005.5397	KCPSLTQTK
1047-1072	2954.2212	TCTTNVDCQVSCWGDYGVCD ESVWKR
1075-1083	1041.6415	TRSILVQPK
1077-1085	969.6091	SILVQPKGK
1086-1101	1644.7832	GKACPSLTQTDACPPR
1088-1104	1805.7979	ACPSLTQTDACPPRDCK
1102-1120	2030.8443	DCKVSGWSQYGACDANGNR
1105-1122	1954.8573	VSGWSQYGACDANGNRNR
1123-1131	1087.6218	TRQIVTDVR
1125-1135	1129.6324	QIVTDVRGGGK
1172-1193	2331.1874	SRSIQVQPAYGGTACPPLTQ TR
1174-1197	2534.2490	SIQVQPAYGGTACPPLTQTR SCQK
1194-1216	2602.1370	SCQKCIVSDWSDWSICTADT PTR
1198-1218	2459.1118	CIVSDWSDWSICTADTPTRF R

Table 6.2 Theoretical tryptic digest of the predicted Vsv protein using the PeptideMass software (http://kr.expasy.org/tools/peptide-mass.html). In this theoretical digest one cleavage site was missed. Fragments between 750 and 3500 Da are shown. The mono-isotopic masses giving peptide masses as [M+H+] are displayed. All cysteins were set as not modified, the methionines were set as not oxidised. The two protein fragments that have been confirmed using MALDI-TOF MS are shaded.

Position	Mass	Peptide sequence
1219-1250	3378.6780	TRVVVQQPPPPSTCGNSDTD DVAVSLQCPVLR
1251-1280	3387.4351	DVQPCDAINCTVTDWGDWGD CNPDTYVKTR
1281-1290	1153.6800	NRTIVQPAVR
1379-1388	1172.6382	ERSIVQDAVR
1381-1400	2016.0290	SIVQDAVRGGAACDVLAQSR
1389-1413	2620.1952	GGAACDVLAQSRTCPPVNCE LSDWK
1401-1421	2393.0722	TCPPVNCELSDWKAWQVCDA K
1414-1425	1307.6412	AWQVCDAKTGTK
1428-1433	761.4264	TRDITR
1430-1437	972.5221	DITRSPQR
1625-1633	1015.5643	TRSATQVPR
1627-1654	2826.2967	SATQVPRHGGADCPALNSTI ACDPVDCK
1655-1671	1891.8530	VSDWSDYGACDAVTFKK
1724-1731	944.5523	RTITTQPK
1774-1792	1893.9599	TRSVVQPALYGGSACGATR
1793-1820	2886.2313	EEVPCGAVNCVVGNWTGWSS CDASCGKK
1823-1829	888.5010	TRQVTQR
1871-1892	2300.3158	TLTRPVLQAALYGGKACPVT IK
1886-1894	960.5546	ACPVTIKTK
1893-1918	2829.1913	TKSCDPVDCTVNDWQSWGVC NADTGK
1895-1919	2728.1436	SCDPVDCTVNDWQSWGVCNA DTGKK
1924-1935	1268.6303	TVKQGDLYGGCK
2020-2029	1156.6797	TRTVLQPSVR
2067-2077	1237.7739	TRSRPIIVAPK
2119-2124	772.4675	RDILQK
2120-2126	859.4883	DILQKDK
2125-2143	1972.8197	DKYGGACCPTLTDSAPCDR
2144-2166	2475.0737	VDCVMNDFGPWTSCDATTGA KTR
2167-2196	3143.4594	TRTVQVAPLYGGSACPSTTE QGFCNPIDCK
2197-2215	2000.9494	VSDWSAFGACNTTSGLKTR
2267-2289	2395.1706	KIIVSPAYGGIDCPCLTETS PCK
2290-2310	2372.0468	MPTPTNCTVTAWSDWTKCSS R
2307-2314	853.3945	CSSRSGTR
2311-2318	915.4755	SGTRTHTR
2366-2387	2637.1418	TRVILDDPYTCSDFCEATMD WR

Table 6.2 - continued

6.3.3. N-terminal sequencing of the Vsv protein

N-terminal sequencing of the putative Vsv protein after 1D-gel electrophoresis and blotting onto a PVDF membrane was carried out. Fig. 6.2 shows the spectra obtained in cycles one to four. The first two amino acid residues correspond to the predicted amino acid sequence of the Vsv protein. The remaining amino acid residues could not be identified, as high background derived from acid hydrolysis of the large protein (approx. 260 kDa) during sequencing was present.

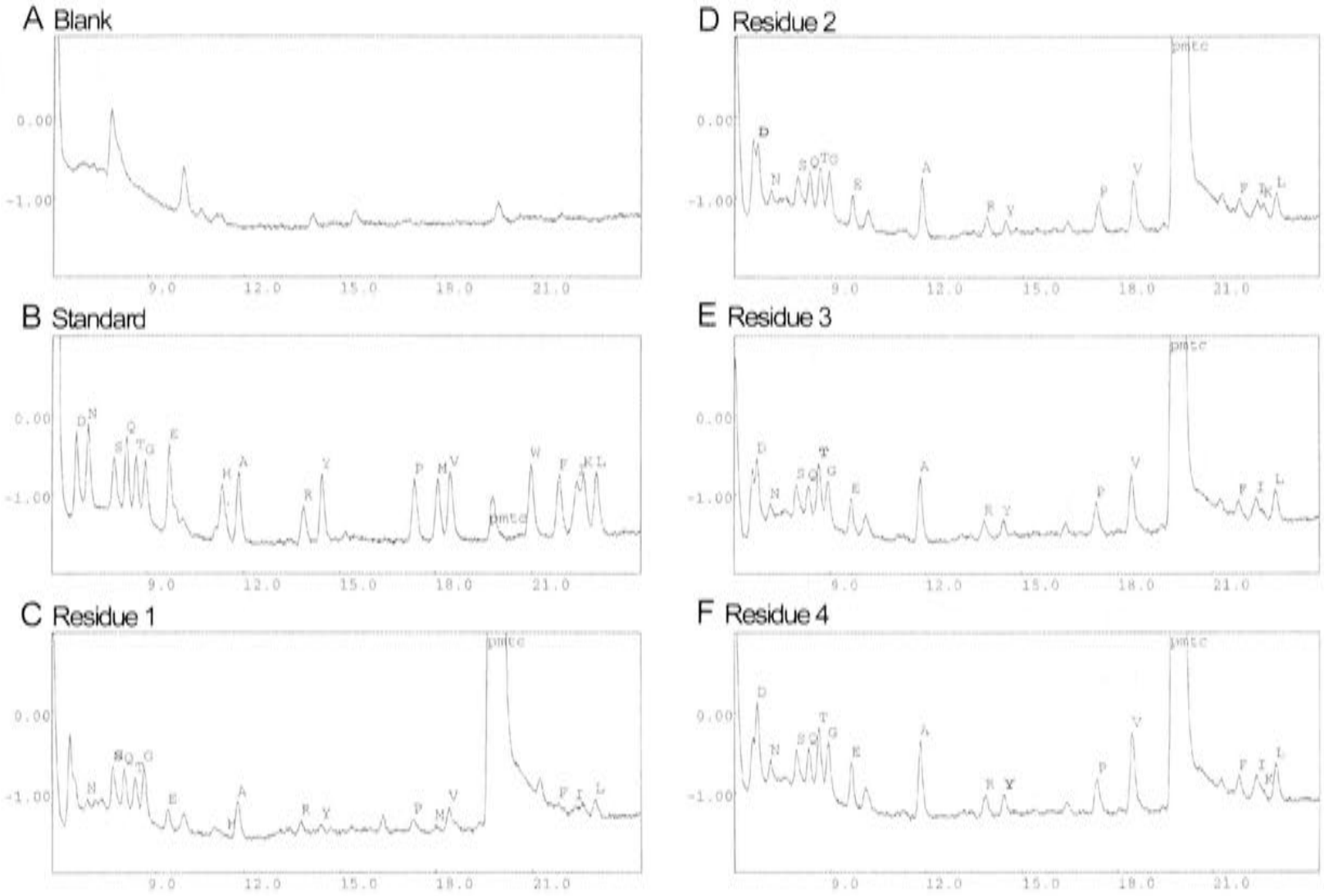

Fig. 6.2 Results obtained during N-terminal sequencing of the 1D protein band containing the Vsv protein. A and B show the results obtained during the calibration of the instrument, C – F show the intensities of the amino acids obtained in the first four cycles as indicated.

6.3.4. Partial purification of the Vsv protein

Ammonium sulphate precipitation (20%, 40%, 60%, and 80%) was carried out to partially purify the Vsv protein for internal sequencing. A polypeptide of the appropriate molecular weight was identified by the monoclonal antibody in the 40 and 60% fractions. It could not be detected in the 20 and 80% fractions (data not shown).

6.4 Discussion

The current chapter was aimed at verifying the identity of the Vsv protein. In a first attempt, an immunological approach was used unsuccessfully. The putative monospecific polyclonal antibodies did not recognise the contents of the ventral vesicles in *P. cinnamomi*, but contents of the large peripheral vesicles. It is known that the contents of the large peripheral vesicles constitute one of the predominant antigens in *P. cinnamomi*, as can be deduced from the number of monoclonal antibodies towards it. As reported in Hardham et al. (1991b), there are six monoclonal antibodies available towards antigens in the large peripheral vesicles in *P. cinnamomi*. All these antibodies were obtained using a routine immunisation of mice with 95-98% zoospores and 2-5% cysts fixed in 2% glutaraldehyde in 50 mM PIPES. The immunoglobulin G polyclonal antibodies used in the present study were generated in rabbits, initially using the same fixation protocol as described for the production of the Lpv antibodies. For the three booster immunisations in the study by Marshall et al. (2001), 50% zoospores and 50% cysts were fixed in 0.2% glutaraldehyde and 4% formaldehyde in 50 mM PIPES buffer. Screening of the polyclonal serum in indirect immunofluorescence assays revealed that antibodies that label the contents of the large peripheral vesicles predominated in the serum (Prof. A.R. Hardham, personal communication). The polyclonal serum was then used in an initial screening of cDNA expression libraries, a method that in the end allowed partial cloning of the *Lpv* genes (Marshall et al. 2001). It is possible, that Lpv antibodies are so abundant in the polyclonal immunoglobulin G fraction that the pre-clearing step employed in the current study was not sufficient to remove them. This could mean that Lpv antibodies stuck non-specifically to the immunopurification membrane and represented the largest fraction of the presumed monospecific polyclonal Vsv antibodies. This theory is supported by the fact that the nucleotide sequence of *Lpv* and *Vsv* genes shows no homology at all (not shown). It might be necessary to include several pre-clearing steps using membranes coated with the fusion-protein encoded by *Lpv* cDNA clones, for example, *Lpv18* (Marshall et al. 2001).

Both, MALDI-TOF MS and N-terminal sequencing of the putative Vsv protein obtained after 1D-gel electrophoresis were partly successful: in the MALDI-TOF MS the mass of one fragment is identical to the mass of a fragment that was predicted in a theoretical digest of the putative Vsv protein. The peptide masses of two fragments could have resulted from missed cleavage of the Vsv protein during the tryptic digest (compare Fig. 6.1 with Tables 6.1 and 6.2). However, when database searches were carried out with the three candidate peptide masses, some known or predicted proteins with identical peptide fragment masses were found meaning the protein could not be identified unambiguously. In the database search the number of missed cleavages had to be set at one which meant that the probability of chance-hits increased. In order to identify any protein, at least four to five empirical peptide masses should match with the theoretical data (reviewed by Nyman 2001). A high background was present in the graph in Fig. 6.1. Although only pico- to femtomole amounts of protein are necessary for MALDI-TOF MS (reviewed in Cait and Kent 1992 or Corthals et al. 2000), it is possible that the amount of protein was insufficient considering the large apparent molecular weight of over 260 kDa (see Chapter 5). In addition, chemical reactions of protein fragments with the Coomassie Brilliant Blue stain or unpolymerised acrylamide could have lead to a further decrease in the amount of protein available for analysis and to low signal-to-noise ratios (for reviews see Gross and Strupat 1998, Herbert et al. 2001 or Hamdan et al. 2001). The protein could not be concentrated and purified using 2D-gel electrophoresis since it does not migrate in the first dimension (not shown). This phenomenon is well known for most proteins with a molecular weight larger than approx. 100 kDa or for hydrophobic proteins (reviewed in Corthals et al. 2000). Other purification methods have so far involved attempts of immunopurification or AS precipitation. The latter approach gave some positive results in the fractions containing 40 and 60% AS, and could be improved. The sample that had been obtained after 1D-gel electrophoresis was subjected to MS. The sample could have consisted of a mixture of proteins, and fragments resulting from contaminating proteins could have prevailed in the sample obscuring the signal from the fragments of the Vsv protein. N-terminal sequencing revealed that the first two amino acids are in accordance with sequence prediction, but already the third amino acid residue

could not be recognised with certainty. From the spectra in Fig. 6.2 it can be concluded that more than one protein was present in the sample. The inherent artefact of acid hydrolysis of the putative Vsv protein could have contributed significantly to the problems encountered during N-terminal sequencing (Dr. P. Milbourne, personal communication).

Although the results from the immunological approach are in contradiction to the expectations, the results obtained using MS and N-terminal protein sequencing are in accordance with the assumption that the *Vsv* gene sequenced in the course of this PhD indeed encodes the Vsv protein. However, direct evidence was lacking until very recently polyclonal antibodies were raised towards a synthetic peptide consisting of the 29 amino acid residues of the predicted Vsv protein after removal of the N-terminal signal sequence. These antibodies show clear labelling of the ventral vesicles in immunofluorescence assays strongly supporting the idea that the *Vsv* gene codes for the putative Vsv protein (Prof. A.R. Hardham, personal communication).

Chapter 7 Towards understanding *Phytophthora* pathogenicity

7.1 The characterisation of *Phytophthora* zoospore components

Phytophthora species are notorious plant pathogens that can infect a wide variety of plant species. For example, *P. cinnamomi* causes the Jarrah dieback and has a major impact on Western Australian forests by infecting and destroying susceptible plant species. *P. cinnamomi*, as all other *Phytophthora* species, can produce asexual motile spores termed zoospores. Zoospores are thought to constitute the main inoculum under conditions favouring disease. They are built for short-range dispersal in a wet environment. Zoospore motility is dependent on the presence of tubular appendages on their anterior flagellum (Cahill et al. 1996). These appendages - called tripartite tubular hairs or mastigonemes – could therefore also be crucial for *Phytophthora* pathogenicity. In addition to their specialised surface appendices, zoospores contain everything needed for the early stages of infection of plants including four types of storage vesicles. The contents of three types of vesicles are secreted. The timing of secretion as well as their localisation in the very early stages of infection indicates a role in early plant infection. Detailed knowledge about the zoospore components mentioned above could contribute significantly to our understanding of their biology and the factors involved in pathogenicity. For example, in a previous study, a monoclonal antibody directed towards the tubular shaft of the mastigonemes of *P. nicotianae* zoospores was generated (Robold and Hardham 1998). This antibody, Pn14B7, recognised its epitope on immunoblots of denatured protein gels, and for the first time, information about the biochemistry of a protein present in these flagellar appendages became available. However, the sequence of the gene encoding the protein is still to be determined. In the present study, monoclonal antibodies were employed to gather information about (1) the localisation of certain components within the zoospores, (2) the developmental stages in which certain zoospore components

are present during the asexual life cycle of *P. nicotianae*, (3) the biochemistry of some of the zoospore components, and (4) the identification of a partial cDNA clone encoding part of the putative zoospore adhesive in *P. cinnamomi*.

An attempt was made using monoclonal antibody Pn14B7 to identify a cDNA clone encoding its antigen. Unfortunately, the identified clone turned out to be a false positive. In order to optimise the chance to obtain a positive cDNA clone that indeed encodes the Pn14B7 antigen, a developmental time course and subsequent immunofluorescent labelling of the samples should be carried out in order to determine at what time most of the antigen is produced. After that, a new cDNA library from mRNA obtained from several time points before that should be made and screened with the antibody. Additionally, raising new monoclonal antibodies directed towards denatured antigens from a preparation enriched in mastigonemes that can be used for immunological screening of an approproiate cDNA expression library could be an option to gain knowledge about the molecular bases of mastigoneme components.

The first approach to characterise *Phytophthora* vesicle proteins utilised monoclonal antibodies raised against components present in *L. giganteum* zoospores. The aims of the approach were to find out whether immunologically related antigens are present in *P. cinnamomi*. However, none of the monoclonal antibodies labelled *P. cinnamomi* large peripheral, dorsal or ventral vesicles, and an alternative approach to the characterisation to the characterisation of these presumptive pathogenicity factors was followed.

Monoclonal antibodies directed towards a wide variety of zoospore antigens were produced employing a technique known as co-immunisation. This protocol enhanced the production of antibodies directed towards components present in zoospores but absent in vegetative hyphae. A high proportion of the antibodies recognise vesicle antigens in *P. nicotianae* zoospores including antigens contained in the large peripheral, dorsal, and ventral vesicles present in the zoospore cortex. This was the first time antibodies that labelled the dorsal vesicle contents in *P. nicotianae* zoospores had been obtained. Monoclonal antibodies directed towards the ventral vesicle contents not only reacted with their antigen in *P. nicotianae* – the organism used for generating the antibodies – but also with the *Vsv* homologues in the related oomycete

species *Py. aphanidermatum* in immunofluorescence and immunogold assays. In addition to the immunofluorescence and immunogold assays, some monoclonal antibodies also reacted on immunoblots of zoospore proteins in the case of *P. cinnamomi*. Ventral vesicles have been implicated in containing the adhesive for incipient *P. cinnamomi* cysts (Hardham and Gubler 1990), and therefore, it was one of the major aims of this project to find tools for their characterisation. The biochemical data obtained with the new monoclonal antibodies were consistent with the data first discovered with *P. cinnamomi* monoclonal antibody Vsv-1 (Hardham and Gubler 1990). Additionally, the new monoclonal antibodies yielded information about the *Vsv* homologue in *P. nicotianae*, where the protein is slightly larger than in *P. cinnamomi*. After the initial characterisation of the new monoclonal antibodies, their use in screening a cDNA expression library led to the sequencing of the gene encoding *Vsv* in *P. cinnamomi*, demonstrating the usefulness and versatility of the immunoscreening approach. Therefore, it is well worth using monoclonal antibodies directed towards antigens in plant pathogens not only to gain information about the (sub-)cellular localisation or biochemical data of their antigens, but also to gather molecular data. The *Vsv* sequence information is most important for the further analysis of the Vsv protein as will be described below.

7.2 Future prospects regarding the Vsv protein

7.2.1. What questions need to be addressed first?

The cloning and sequencing of the *Vsv* gene opened up a vast number of experiments that can be carried out. Hence, the first question one needs to ask is "Which experiments first?" No doubt, exciting times which promise to give us deeper insight into oomycete infection strategies lie ahead of us. In my opinion, the most urgent experiments are concerned with the pattern of expression of the *Vsv* gene during important developmental stages. Northern blotting was not successful, so reverse transcriptase PCR at various time points during the development of zoospores could be used to determine when the gene is

expressed. Quantitative analysis could then be carried out with real-time PCR. In-situ hybridisation is another possibility that could be tested. However, all these approaches will have to be optimised for the *Vsv* gene.

7.2.2. Adhesion in *Phytophthora* zoospores: pathogenicity or virulence factor?

During the course of my thesis I have gained knowledge about the molecular basis of the putative adhesive in *P. cinnamomi*. However, the question of the function and necessity of the Vsv protein during the early stages of infection needs to be addressed urgently. Two strategies should be tested: adhesion assays and gene silencing. Preliminary adhesion assays using the monoclonal antibodies Vsv-1 and Pn-Vsv-3 have already been carried out during the course of this thesis following the protocol described by Gubler et al. (1989). In the pilot experiment the sample order was random and zoospore samples were taken from different plates in order to avoid batch effects. Adhesion of the cysts seemed to be inhibited to the same extend by the Vsv-directed monoclonal antibodies as by control monoclonal antibodies directed towards an unrelated antigen, but the standard deviation of the experiments was large; thus, the results were inconclusive. A better approach to test the function of the Vsv protein would be the transformation of *P. cinnamomi* with a *Vsv* construct that allows silencing of the gene. Since the Vsv protein is only present in hyphae upon the induction of sporulation, in zoosporangia, zoospores, and chlamydospores, a function in non-sporulating hyphae seems unlikely (Dearnaley et al. 1996). Strains with silenced *Vsv* gene expression should therefore be viable at least under non-sporulating conditions. If zoospores could be obtained, the level of their adhesion could be tested in *in-vitro* assays and compared to the level of adhesion obtained by wild type zoospores. Successful transformation systems have been described for several *Phytophthora* species (see chapter 1). However, transformation of *P. cinnamomi* is still considered difficult (Dr. J. Marshall and Dr. W. Shan, personal communication). A faster approach could be the cloning, sequencing, and silencing of the *Vsv* homologue in, for example, *P. infestans*.

7.2.3. What functions do the different modules of the Vsv protein have?

This question is probably one of the most difficult to answer. To tackle the problem, non-adhesive yeasts could be transformed with constructs encoding TS1-like repeats and the resulting transformants could be monitored for a gain of adhesiveness. However, the function of the GTSAY repeats at the N-terminal end and the function of the C-terminal extension will be even more challenging. To test for a role of either of these domains in subcellular localisation of the Vsv protein, GFP constructs of these domains could be made and used for transformation. In the case of a C-terminal motif found in the apicomplexan TSR 1 protein MIC2, a role in gliding motility and invasion of salivary glands has been demonstrated (Kappe et al. 1999). Any other function of these domains would have to be addressed with targeted mutation, an approach that is difficult to carry out in a diploid organism.

7.2.4. Are components other than the Vsv protein stored in the ventral vesicles?

Trying to answer this question would certainly be worthwhile as important information about the functioning of the adhesive might be obtained. It is still unknown why *Phytophthora* incipient cysts are adhesive for only a short period of time (e.g. 3-4 min in the case of *P. cinnamomi*, Gubler et al. 1989). Purification of intact ventral vesicles might be one way to look for components involved in the adhesion mechanism. Differential fractionation, a protocol that needs to be established for the ventral vesicles, would be one approach to try out (e.g. reviewed in Alberts et al. 2002). Two D-gel electrophoresis could be carried out with the purified vesicles, followed by N-terminal or internal protein sequencing. This approach could also give information on molecules involved in the vesicle trafficking, as vesicle membranes would be co-purified in the samples.

7.2.5. Is the *Vsv* gene highly conserved in other Oomycetes?

It would be exciting to find out more about the phylogenetic relationship of various Oomycete species using sequence analysis of the *Vsv* gene. The sequencing of the *P. sojae* genome has recently been completed, so comparison of its *Vsv* homologue will be carried out as soon as possible. In all other oomycetes, sequence information is scarce, but some species could be easily investigated with regard to their *Vsv* homologue since genomic libraries are available. In our laboratory, genomic libraries of *P. nicotianae* are available, so one of the first experiments would involve the cloning and sequencing the *Vsv* homologue in *P. nicotianae*. For this, a genomic library could be screened with the λgt11-Vsv insert and positive clones purified and analysed. Genomic libraries of *P. infestans* are also available, so the corresponding homologue should also be easily obtained. In the case of other Oomycete species, for example, *Py. aphanidermatum* or *S. ferax*, cultures are already available. Partial genomic libraries could be made and screened as described for *P. nicotianae* or reverse-transcriptase PCR of the appropriate developmental stages could be carried out and positive clones or PCR products sequenced. Although analysis of the preliminary genome database of the diatome *T. pseudonana* revealed no homologue of the *Phytophthora Vsv* gene, it would be interesting to see if a *Vsv* homologue is present in other members of the Stramenopile kingdom.

Cloning and sequencing the *Vsv* gene from *P. cinnamomi* represents the first molecular characterisation of an adhesive of a propagule of a fungal or oomycete plant pathogen. The Vsv adhesive shares features in common with one category of animal cell adhesives but similar molecules do not appear to be present in the genomes of the true fungi. Further studies of the mechanisms involved in the function of the Vsv adhesive and its occurrence in other organisms promises to be a worthwhile and exciting endeavour.

Bibliography

Adair, W. S., C. Hwang, *et al.* (1983). "Identification and visualization of the sexual agglutinin from the mating-type plus flagellar membrane of *Chlamydomonas*." Cell **33**(1): 183-193.

Adams, J. C. (2001). "Thrombospondins: multifunctional regulators of cell interactions." Annual Review of Cell and Developmental Biology **17**: 25-51.

Adams, J. C. and R. P. Tucker (2000). "The thrombospondin type 1 repeat (TSR) superfamily: diverse proteins with related roles in neuronal development." Developmental Dynamics **218**: 280-299.

Alberts, B., A. E. Johnson, *et al.* (2002). Molecular biology of the cell. New York, Garland Science.

Altschul, S. F., T. L. Madden, *et al.* (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Research **25**: 3389-3402.

Ambikapathy, J., J. S. Marshall, *et al.* (2002). "The role of proline in osmoregulation in *Phytophthora nicotianae*." Fungal Genetics and Biology **35**: 287-299.

Arrendondo, F., A. Chan, *et al.* (1997). Construction of a BAC contig of *Phytophthora sojae*. 19th Fungal Genetics Conference, 34 Pacific Grove, CA.

Bachmair, A., D. Finley, *et al.* (1986). "In vivo half-life of a protein is a function of its amino-terminal residue." Science **234**(4773): 179-186.

Bailey, A. M., G. L. Mena, *et al.* (1991). "Genetic transformation of the plant pathogens *Phytophthora capsici* and *Phytophthora parasitica*." Nucleic Acids Research **19**: 4273-4278.

Barclay, S. L. and A. M. Smith (1986). "Rapid isolation of monoclonal antibodies specific for cell surface differentiation antigens." Proceedings of the National Academy of Sciences of the United States of America **83**: 4336-4340.

Barr, D. J. S. (1983). The zoosporic grouping of plant pathogens. Entity or non-entity? Zoosporic Plant Pathogens. A Modern Perspective. London, Academic Press: 43-83.

Bartnicki-Garcia, S. and V. O. Sing (1987). Adhesion of zoospores of *Phytophthora* to solid surfaces. Zoosporic Fungi in Teaching & Research. M. S. Fuller and A. Jaworski. Athens, GA, Southeastern Publishing Corporation: 279-283.

Bartnicki-Garcia, S. and M. C. Wang (1983). Biochemical aspects of morphogenesis in *Phytophthora*. *Phytophthora*. Its Biology, Taxonomy, Ecology, and Pathology. D. C. Erwin, S. Bartnicki-Garcia and P. Tsao. St Paul, Minnesota, American Phytopathological Society: 121-137.

Beakes, G. W. (1987). Oomycete phylogeny: ultrastructural perpectives. Evolutionary Biology of the Fungi. A. D. M. Rayner, C. M. Brasier and D. Moore. Cambridge, Cambridge University Press: 405-421.

Beakes, G. W. (1989). Oomycete fungi: their phylogeny and relationship to chromophyte algae. The Chromophyte Algae: Problems and Perspectives. J. C. Green, B. S. C. Leadbeater and W. L. Diver. Oxford, Clarendon Press: 325-342.

Beckett, A., J. A. Tatnell, *et al.* (1990). "Adhesion and pre-invasion behaviour of urediniospores of *Uromyces viciae-fabae* during germination on host and synthetic surfaces." Mycological Research **94**: 865-875.

Berbee, M. L. and J. L. Kerwin (1993). "Ultrastructural and light microscopic localization of carbohydrates and peroxidase/catalases in *Lagenidium giganteum* zoospores." Mycologia **85**: 734-743.

Bimpong, C. E. and C. J. Hickman (1975). "Ultrastructural and cytochemical studies of zoospores, cysts, and germinating cysts of *Phytophthora palmivora*." Canadian Journal of Botany **53**: 1310-1327.

Bjellqvist, B., B. Basse, *et al.* (1994). "Reference points for comparisons of two-dimensional maps of proteins from different human cell types defined in a pH scale where isoelectric points correlate with polypeptide compositions." Electrophoresis **15**(3-4): 529-539.

Bjellqvist, B., G. J. Hughes, *et al.* (1993). "The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences." Electrophoresis **14**(10): 1023-1031.

Bloodgood, R. A. (2000). "Protein targeting to flagella of trypanosomatid protozoa." Cell Biology International **24**(12): 857-862.

Bloodgood, R. A. and N. L. Salomonsky (1989). "Use of a novel Chlamydomonas mutant to demonstrate that flagellar glycoprotein movements are necessary for the expression of gliding motility." Cell Motility and the Cytoskeleton **13**: 1-8.

Blum, H., H. Beier, *et al.* (1987). "Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels." Electrophoresis **8**(2): 93-99.

Boekel, C. and N. H. Brown (2002). "Integrins in development: moving on, responding to, and sticking to the extracellular matrix." Developmental Cell **3**(3): 311-321.

Bottin, A., L. Larche, *et al.* (1999). "Green fluorescent protein (GFP) as gene expression reporter and vital marker for studying development and microbe-plant interaction in the tobacco pathogen *Phythphthora parasitica* var. *nicotianae*." FEMS Microbiology Letters **176**(1): 51-56.

Bourke, P. M. (1964). "Emergence of potato blight." Nature **203**: 805-808.

Brandhorst, T. T., M. Wuethrich, *et al.* (1999). "Targeted gene disruption reveals an adhesin indispensable for pathogenicity of *Blastomyces dermatitidis*." Journal of Experimental Medicine **189**(8): 1207-16.

Braun, E. J. and R. J. Howard (1994). "Adhesion of fungal spores and germlings to host plant surfaces." Protoplasma **181**: 202-212.

Braun, J. E. and H. J. Neusser (2002). "Threshold photoionization in time-of-flight mass spectrometry." Mass Spectrometry Reviews **21**(1): 16-36.

Burr, A. W. and G. W. Beakes (1994). "Characterization of zoospore and cyst surface structure in saprophytic and fish pathogenic *Saprolegnia* species (oomycete fungal protists)." Protoplasma **181**: 142-163.

Caesar-TonThat, T. C. and L. Epstein (1991). "Adhesion-reduced mutants and the wild-type *Nectria haematococca*: an ultrastructural comparison of the macroconidial walls." Experimental Mycology **15**: 193-205.

Cahill, D. M., M. Cope, *et al.* (1996). "Thrust reversal by tubular mastigonemes: immunological evidence for a role of mastigonemes in forward motion of zoospores of *Phytophthora cinnamomi*." Protoplasma **194**: 18-28.

Callow, J. A., S. A. Crawford, *et al.* (2000b). "The application of atomic force microscopy to topographical studies and force measurements on the secreted adhesive of the green alga *Enteromorpha*." Planta **211**: 641-647.

Callow, M. E., J. A. Callow, *et al.* (2000a). "Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores." Applied and Environmental Microbiology **66**(8): 3249-3254.

Callow, M. E., S. Crawford, *et al.* (2001). "Brefeldin A affects adhesion of zoospores of the green alga *Enteromorpha*." Journal of Experimental Botany **52**(360): 1409-1415.

Cameron, J. N. and M. J. Carlile (1978). "Fatty acids, aldehydes and alcohols as attractants for zoospores of *Phytophthora palmivora*." Nature **271**: 448-449.

Canut, H., A. Carrasco, *et al.* (1998). "High affinity RGD-binding sites at the plasma membrane of *Arabidopsis thaliana* links the cell wall." Plant Journal **16**(1): 63-71.

Carruthers, V. B. and L. D. Sibley (1997). "Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts." European Journal of Cell Biology **73**: 114-123.

Celerin, M., A. W. Day, *et al.* (1995). "A glycosylation pattern that is unique to fimbriae from the taxon Microbotryales." Canadian Journal of Microbiology **41**: 452-460.

Chaffin, W. L., J. L. Lopez-Ribot, *et al.* (1998). "Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression." Microbiology and Molecular Biology Reviews **62**(1): 130-180.

Chait, B. T. and S. B. Kent (1992). "Weighing naked proteins: practical, high-accuracy mass measurement of peptides and proteins." Science **257**(5078): 1885-1894.

Chambers, S. M., A. R. Hardham, *et al.* (1995). "In planta immunolabelling of three types of peripheral vesicles in cells of *Phytophthora cinnamomi* infecting chestnut roots." Mycological Research **99**: 1281-1288.

Chaurand, P. and R. M. Caprioli (2002). "Direct profiling and imaging of peptides and proteins from mammalian cells and tissue sections by mass spectrometry." Electrophoresis **23**(18): 3125-3135.

Chiquet-Ehrismann, R. (1993). Tenascin. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 93-95.

Ciechanover, A. and A. L. Schwartz (1989). "How are substrates recognized by the ubiquitin-mediated proteolytic system?" Trends in Biochemical Science **14**(12): 483-488.

Clement, J. A., T. M. Butt, *et al.* (1993a). "Characterization of the extracellular matrix produced in vitro by urediniospores and sporelings of *Uromyces viciae-fabae*." Mycological Research **97**: 594-602.

Clement, J. A., S. G. Martin, *et al.* (1993b). "Germination and the role of the extracellular matrix in adhesion of urediniospores of *Uromyces viciae-fabae* to synthetic surfaces." Mycological Research **97**: 585-593.

Clement, J. A., R. Porter, *et al.* (1994). "The role of hydrophobicity in attachment of urediniospores and sporelings of *Uromyces viciae-fabae*." Mycological Research **98**: 1217-1228.

Cole, D. G., D. R. Diener, *et al.* (1998). "*Chlamydomonas* Kinesin-II-dependent Intraflagellar Transport (IFT): IFT particles contain proteins required for ciliary assembly in *Caenorhabditis elegans* sensory neurons." Journal of Cell Biology **141**(4): 993-1008.

Cooke, D. E., Drenth, A., *et al.* (2000). "A molecular phylogeny of *Phytophthora* and related oomycetes." Fungal Genetics and Biology **30**(1): 17-32.

Cope, M. and A. R. Hardham (1994). "Synthesis and assembly of flagellar surface antigens during zoosporogenesis in *Phytophthora cinnamomi*." Protoplasma **180**: 158-168.

Cope, M., M. C. Webb, *et al.* (1996). "Immunocytochemical comparison of peripheral vesicles in zoospores of *Phytophthora* and *Pythium* species." Mycologia **88**: 523-532.

Corrêa, A., Jr, R. C. Staples, *et al.* (1996). "Inhibition of thigmostimulated cell differentiation with RGD-peptides in *Uromyces* germlings." Protoplasma **194**(1-2): 91-102.

Corrêa, A., Jr. and H. C. Hoch (1995). "Identification of thigmoresponsive loci for cell differentiation in *Uromyces* germlings." Protoplasma **186**: 34-40.

Corthals, G. L., S. P. Gygi, *et al.* (2000). Identification of proteins by mass spectrometry. Proteome research: 2-dimensional gel electrophoresis and identification methods. R. Rabilloud. Heidelberg, Springer Verlag: 197-231.

Cotter, G. and K. Kavanagh (2000). "Adherence mechanisms of *Candida albicans*." British Journal of Biomedial Science **57**: 241-249.

Deahl, K. L., R. W. Groth, *et al.* (1991). "Occurrence of the A2 mating type of *Phytophthora infestans* in potato fields in the United States and Canada." American Potato Journal **68**: 717-726.

Dean, R. A. (1997). "Signal pathways and appressorium morphogenesis." Annual Review of Phytopathology **35**: 211-234.

Dearnaley, J. D. W. and A. R. Hardham (1994). "The Golgi apparatus of *Phytophthora cinnamomi* makes three types of secretory or storage vesicles concurrently." Protoplasma **182**: 75-79.

Dearnaley, J. D. W., J. Maleszka, *et al.* (1996). "Synthesis of zoospore peripheral vesicles during sporulation of *Phytophthora cinnamomi*." Mycological Research **100**(1): 39-48.

Deising, H., R. L. Nicholson, *et al.* (1992). "Adhesion pad formation and the involvement of cutinase and esterases in the attachment of uredospores to the host cuticle." Plant Cell **4**: 1101-1111.

Demets, R., A. M. Tomson, *et al.* (1988). "Cell-cell adhesion in conjugating *Chlamydomonas* gametes: a self-enhancing process." Protoplasma **145**: 27-36.

Dersch, P. (2003). "Molecular and cellular mechanisms of bacterial entry into host cells." Contributions to Microbiology **10**: 183-209.

Dick, M. W. (2001). Straminipilous fungi : systematics of the peronosporomycetes, including accounts of the marine straminipilous protists, the plasmodiophorids, and similar organisms. Dordrecht ; Boston, Kluwer Academic Publishers.

Didier, R., I. Creveaux, *et al.* (2000). "SCO-spondin and RF-Glyl: two designations for the same glycoprotein secreted by the subcommissural organ." Journal of Neuroscience Research **61**(5): 500-507.

Dirksen, L. B., T. Proft, *et al.* (1996). "Sequence analysis and characterization of the hmw gene cluster of *Mycoplasma pneumoniae*." Gene **171**(1): 19-25.

Domnas, A., S. Jaronski, *et al.* (1986). "The zoospore and flagellar mastigonemes of *Lagenidium giganteum* (Oomycetes, Lagenidiales)." Mycologia **78**(5): 810-817.

Drenth, A., S. B. Goodwin, *et al.* (1993b). "Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms." Phytopathology **83**: 1087-1092.

Drenth, A., E. M. Jahnssen, *et al.* (1995). "Formation and survival of oospores of *Phytophthora infestans* under natural conditions." Plant Pathology **44**: 86-94.

Drenth, A., L. J. Turkensteen, *et al.* (1993a). "The occurence of the A2 mating type of *Phytophthora infestans* in The Netherlands; significance and consequences." Netherlands Journal of Plant Pathology **99**: 57-67.

Durso, L., L. P. J. Lehnen, *et al.* (1993). "Characteristics of extracellular adhesions produced during *Saprolegnia ferax* secondary zoospore encystment and cystospore germination." Mycologia **85**: 744-755.

El Zein, L., H. Omran, *et al.* (2003). "Lateralization defects and ciliary dyskinesia: lessons from algae." Trends in Genetics **19**(3): 162-167.

Engvall, E. (1993). Laminin. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 66-68.

Epstein, L., Y. Kwon, *et al.* (1994). "Genetic and biochemical characterization of *Nectria haematococca* strains with adhesive and adhesion-reduced macroconidia." Applied and Environmental Microbiology **60**(2): 524-530.

Epstein, L., L. B. Laccetti, *et al.* (1987). "Cell-substratum adhesive protein involved in surface contact responses of the bean rust fungus." Physiological and Molecular Plant Pathology **30**: 373-388.

Erwin, D. C. and O. K. Ribeiro (1996). *Phytophthora* diseases worldwide. St. Paul, Minnesota, APS Press.

Estrada-Garcia, M. T., J. A. Callow, *et al.* (1990a). "Monoclonal antibodies to the adhesive cell coat secreted by *Pythium aphanidermatum* zoospores recognise 200 x 10^3Mr glycoproteins stored within large peripheral vesicles." Journal of Cell Science **95**: 199-206.

Estrada-Garcia, M. T., J. R. Green, *et al.* (1989). "Monoclonal antibodies to cell surface components of zoospores and cysts of the fungus *Pythium aphanidermatum* reveal species-specific antigens." Experimental Mycology **13**: 348-355.

Estrada-Garcia, T., T. C. Ray, *et al.* (1990b). "Encystment of *Pythium aphanidermatum* zoospores is induced by root mucilage polysaccharides, pectin and a monoclonal antibody to a surface antigen." Journal of Experimental Biology **41**: 693-699.

Fenn, J. B., M. Mann, *et al.* (1989). "Electrospray ionization for mass spectrometry of large biomolecules." Science **246**(4926): 64-71.

Ferris, P. J., J. P. Woessner, *et al.* (1996). "A sex recognition glycoprotein is encoded by the plus mating-type gene fus1 of *Chlamydomonas reinhardtii*." Molecular Biology of the Cell **7**(8): 1235-1248.

Fisher, A. G., C. M. Bunce, *et al.* (1982). "Studies of human myeloid antigens using monoclonal antibodies and variant lines from the promyeloid cell line HL60." Clinical Experiments in Immunology **50**: 374-381.

Gabor, B. K., E. T. O'Gara, *et al.* (1993). "Specificities of monoclonal antibodies to *Phytophthora cinnamomi* in two rapid diagnostic assays." Plant Disease **77**: 1189-1197.

Gardiner, R., C. Podgorski, *et al.* (1982). "Serological studies on the fimbriae of yeasts and yeastlike species." Botanical Gazette **143**: 534-541.

Gardiner, R. B. and A. W. Day (1988). "Surface proteinaceous fibrils (fimbriae) on filamentous fungi." Canadian Journal of Botany **66**: 2474-2484.

Gautam, Y., D. M. Cahill, *et al.* (1999). "Development of a quantitative immunodipstick assay for *Phytophthora nicotianae*." Food and Agricultural Immunology **11**: 229-242.

Giannini, J. L., J. S. Holt, *et al.* (1988). "Isolation of sealed plasma membrane vesicles from *Phytophthora megasperma* f. sp. *glycinea*. I. Characterization of proton pumping and ATPase activity." Archives of Biochemistry and Biophysics **265**(2): 337-345.

Gisi, U. and Y. Cohen (1996). "Resistance to phenylamide fungicides - a case study with *Phytophthora infestans* involving mating type and race structure." Annual Review of Phytopathology **34**: 549-572.

Gobron, S., I. Creveaux, *et al.* (1999). "SCO-spondin is evolutionarily conserved in the central nervous system of the chordate phylum." Neuroscience **88**(2): 655-664.

Goding, J. W. (1983). Monoclonal Antibodies: Principles and Practice. London, Academic Press.

Golumbeski, J., G.S. and R. L. Dimond (1986). "The use of tolerization in the production of monoclonal anitbodies against minor antigenic determinants." Analytical Biochemistry **154**: 373-381.

Goncalves-Mendes, N., D. Simon-Chazottes, *et al.* (2003). "Mouse SCO-spondin, a gene of the thrombospondin type 1 repeat (TSR) superfamily expressed in the brain." Gene **312**: 263-270.

Gonda, D. K., A. Bachmair, *et al.* (1989). "Universality and structure of the N-end rule." Journal of Biological Chemistry **264**(28): 16700-16712.

Goodenough, U. W. (1989). "Cyclic AMP enhances the sexual agglutinability of *Chlamydomonas flagella*." Journal of Cell Biology **109**(1): 247-252.

Gorman, J. J., T. P. Wallis, *et al.* (2002). "Protein disulfide bond determination by mass spectrometry." Mass Spectrometry Reviews **21**(3): 183-216.

Gotelli, D. (1974). "The morphology of *Lagenidium callinectes* II. zoosporogenesis." Mycologia **66**: 846-858.

Gross, J. and K. Strupat (1998). "Matrix-assisted laser desorption/ionisation mass spectrometry applied to biological macromolecules." TRAC-Trends in Analytical Chemistry **17**(8-9): 470-484.

Grove, S. N. and C. E. Bracker (1978). "Protoplasmic changes during zoospore encystment and cyst germination in *Pythium aphanidermatum*." Experimental Mycology **2**: 51-98.

Gruenheid, S. and B. B. Finlay (2003). "Microbial pathogenesis and cytoskeletal function." Nature **422**(6933): 775-781.

Gubler, F. and A. R. Hardham (1988). "Secretion of adhesive material during encystment of *Phytophthora cinnamomi* zoospores, characterized by immunogold labelling with monoclonal antibodies to components of peripheral vesicles." Journal of Cell Science **90**: 225-235.

Gubler, F. and A. R. Hardham (1990). "Protein storage in large peripheral vesicles in *Phytophthora* zoospores and its breakdown after cyst germination." Experimental Mycology **14**: 393-404.

Gubler, F. and A. R. Hardham (1991). The fate of peripheral vesicles in zoospores of *Phytophthora cinnamomi* during infection of plants. Electron Microscopy of Plant Pathogens. K. Mendgen and D.-E. Lesemann. Berlin, Springer-Verlag: 197-210.

Gubler, F., A. R. Hardham, *et al.* (1989). "Characterising adhesiveness of *Phytophthora cinnamomi* zoospores during encystment." Protoplasma **149**: 24-30.

Gubler, F., P. P. Jablonsky, *et al.* (1990). "Localization of calmodulin in flagella of zoospores of *Phytophthora cinnamomi*." Protoplasma **155**: 233-238.

Hamdan, M., M. Galvani, *et al.* (2001). "Monitoring 2-D gel-induced modifications of proteins by MALDI-TOF mass spectrometry." Mass Spectrometry Reviews **20**(3): 121-141.

Hamer, J. E., R. J. Howard, *et al.* (1988). "A mechanism for surface attachment in spores of a plant pathogenic fungus." Science **239**: 288-290.

Hamer, J. E., B. Valent, *et al.* (1989). "Mutations at the SMO genetic locus affect the shape of diverse cell types in the rice blast fungus." Genetics **122**(2): 351-361.

Hamilton, A. J., L. Jeavons, *et al.* (1999). "Recognition of fibronectin by *Penicillium marneffei* conidia via a sialic acid-dependent process and its relationship to the interaction between conidia and laminin." Infection and Immunity **67**: 5200-5205.

Hanahan, D. (1985). Techniques for transformation of *E. coli*. DNA Cloning. A Practical Approach. D. M. Glover. Oxford, IRL Press. **1:** 109-135.

Hardham, A. R. (1985). "Studies on the cell surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*: the influence of fixation on patterns of lectin binding." Journal of Histochemistry and Cytochemistry **33**: 110-118.

Hardham, A. R. (1987a). "Microtubules and the flagellar apparatus in zoospores and cysts of the fungus *Phytophthora cinnamomi*." Protoplasma **137**: 109-124.

Hardham, A. R. (1987b). "Ultrastructure and serial section reconstruction of zoospores of the fungus *Phytophthora cinnamomi*." Experimental Mycology **11**: 297-306.

Hardham, A. R. (1995). "Polarity of vesicle distribution in oomycete zoospores: development of polarity and importance for infection." Canadian Journal of Botany **73(Suppl. 1)**: S400-S407.

Hardham, A. R. (2001). Cell biology of fungal infection of plants. The Mycota Vol.VII: Biology of the Fungal Cell. R. J. Howard and N. A. R. Gow. Heidelberg, Springer-Verlag: 91-123.

Hardham, A. R., D. M. Cahill, *et al.* (1994). "Cell surface antigens of *Phytophthora* spores: biological and taxonomic characterization." Protoplasma **181**: 213-232.

Hardham, A. R. and F. Gubler (1990). "Polarity of attachment of zoospores of a root pathogen and pre-alignment of the emerging germ tube." Cell Biology International Reports **14**: 947-956.

Hardham, A. R., F. Gubler, *et al.* (1991a). Ultrastructural and immunological studies of zoospores of *Phytophthora*. *Phytophthora*. J. A. Lucas, R. C. Shattock, D. S. Shaw and L. R. Cooke. Cambridge, Cambridge University Press: 50-69.

Hardham, A. R., F. Gubler, *et al.* (1991b). "A review of methods for the production and use of monoclonal antibodies to study zoosporic plant pathogens." Journal of Microscopy **162**: 305-318.

Hardham, A. R. and E. Suzaki (1986). "Encystment of zoospores of the fungus, *Phytophthora cinnamomi*, is induced by specific lectin and monoclonal antibody binding to the cell surface." Protoplasma **133**: 165-173.

Hardham, A. R., E. Suzaki, *et al.* (1986). "Monoclonal antibodies to isolate-, species- and genus-specific components on the surface of zoospores and cysts of the fungus *Phytophthora cinnamomi*." Canadian Journal of Botany **64**: 311-321.

Harlow, E. and D. Lane, Eds. (1999). Using Antibodies - A Laboratory Manual. New York, Cold Sprig Harbor Laboratory Press, Cold Spring Harbor, NY.

Harper, J. D. I., F. Gubler, *et al.* (1995). "Centrin association with the flagellar apparatus in spores of *Phytophthora cinnamomi*." Protoplasma **188**: 225-235.

Heath, I. B., A. D. Greenwood, *et al.* (1970). "The origin of flimmer in *Saprolegnia*, *Dictyuchus*, *Synura* and *Cryptomonas*." Journal of Cell Science **7**: 445-461.

Herbert, B., M. Galvani, *et al.* (2001). "Reduction and alkylation of proteins in preparation of two-dimensional map analysis: why, when, and how?" Electrophoresis **22**(10): 2046-2057.

Hohl, H. R. and K. Iselin (1984). "Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour." Transactions of the British Mycological Society **83**(3): 529-530.

Holwill, M. E. J. and M. A. Sleigh (1967). "Propulsion by hispid flagella." Journal of Experimental Biology **47**: 267-276.

Hostetter, M. K. (1999). "Integrin-like proteins in *Candida spp.* and other microorganisms." Fungal Genetics and Biology **28**: 135-145.

Hoyer, L. L. (2001). "The ALS gene family of *Candida albicans*." Trends in Microbiology **9**: 176-180.

Hudspeth, D. S. S., S. A. Nadler, *et al.* (2000). "A COX2 molecular phylogeny of the peronosporomycetes." Mycologia **92**(4): 674-684.

Hughes, H. B., R. Carzaniga, *et al.* (1999). "Spore surface glycoproteins of *Colletotrichum lindemuthianum* are recognized by a monoclonal antibody which inhibits adhesion to polystyrene." Microbiology **145**(8): 1927-1936.

Hutter, H., B. E. Vogel, *et al.* (2000). "Conservation and novelty in the evolution of cell adhesion and extracellular matrix genes." Science **287**(5455): 989-994.

Hyde, G. J., S. Lancelle, *et al.* (1991). "Freeze substitution reveals a new model for sporangial cleavage in *Phytophthora*, a result with implications for cytokinesis in other eukaryotes." Journal of Cell Science **100**: 735-746.

Hynes, R. (1993). Fibronectins. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 56-58.

Hynes, R. O. and Q. Zhao (2000). "The evolution of cell adhesion." Journal of Cell Biology **150**(2): F89-96.

Iruela-Arispe, M. L., D. Caprizo, *et al.* (2003). "ADAMTS1: A matrix metalloprotease with angioinhibitory properties." Annals of the New York Academy of Sciences **995**(1): 183-190.

Iwai, H., N. Masaoka, *et al.* (2002). "From the Cover: A pectin glucuronyltransferase gene is essential for intercellular attachment in the plant meristem." Proceedings of the National Academy of Sciences of the United States of America **99**(25): 16319-16324.

Jahn, T. L., M. D. Landman, *et al.* (1964). "The mechanism of locomotion of flagellates. II. function of the mastigonemes of *Ochromonas*." Journal of Protozoology **11**: 291-296.

Jauh, G. Y., K. J. Eckard, *et al.* (1997). "Adhesion of lily pollen tubes on an artificial matrix." Sexual Plant Reproduction **10**(3): 173-180.

Jones, E. B. G. (1994). "Fungal adhesion." Mycological Research **98**: 961-981.

Jones, M. J. and L. Epstein (1989). "Adhesion of *Nectria haematococca* macroconidia." Physiological and Molecular Plant Pathology **35**: 453-461.

Jones, M. J. and L. Epstein (1990). "Adhesion of macroconidia to the plant surface and virulence of *Nectria haematococca*." Applied and Environmental Microbiology **56**: 3772-3778.

Judelson, H. S., M. D. Coffey, *et al.* (1993a). "Transformation of the oomycete pathogen *Phytophthora megasperma* f. sp. *glycinea* occurs by DNA integration into single or multiple chromosomes." Current Genetics **23**: 211-218.

Judelson, H. S., R. Dudler, *et al.* (1993b). "Expression and antisense inhibition of transgenes in *Phytophthora infestans* is modulated by choice of promoter and position effects." Gene **133**(1): 63-69.

Judelson, H. S., B. M. Tyler, *et al.* (1991). "Transformation of the Oomycete pathogen, *Phytophthora infestans*." Molecular Plant-Microbe Interactions **4**: 602-607.

Kamoun, S., P. Hraber, *et al.* (1999). "Initial assessment of gene diversity for the oomycete pathogen *Phytophthora infestans* based on expressed sequences." Fungal Genetics and Biology **28**: 94-106.

Kappe, S., T. Bruderer, *et al.* (1999). "Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites." Journal of Cell Biology **147**(5): 937-944.

Karas, M. and F. Hillenkamp (1988). "Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons." Analytical Chemistry **60**(20): 2299-2301.

Kerwin, J. L., L. James, *et al.* (1997). Fungi: Oomycetes and Chytridiomycetes. Manual of Techniques in Insect Pathology. L. A. Lacey. New York, Academic Press: 251-268.

Khew, K., L. and G. A. Zentmyer (1973). "Chemotactic response of zoospores of five species of *Phytophthora*." Phytopathology **63**: 1511-1517.

Klein, B. S. (2000). "Molecular basis of pathogenicity in *Blastomyces dermatitidis*: the importance of adhesion." Current Opinion in Microbiology **3**: 339-343.

Knox, J. P. (1992). "Cell adhesion, cell separation and plant morphogenesis." The Plant Journal **2**(2): 137-141.

Koehler, G. and C. Milstein (1975). "Continuous cultures of fused cells secreting antibody of predefined specificity." Nature **256**(5517): 495-497.

Koomen, J. M., W. K. Russell, *et al.* (2002). "Accurate mass measurement of DNA oligonucleotide ions using high-resolution time-of-flight mass spectrometry." Journal of Mass Spectrometry **37**(4): 357-371.

Kozminski, K., K. Johnson, *et al.* (1993). "A motility in the eukaryotic flagellum unrelated to flagellar beating." Proceedings of the National Academy of Sciences of the United States of America **90**(12): 5519-5523.

Kuno, K., N. Kanada, *et al.* (1997). "Molecular cloning of a gene encoding a new type of metalloproteinase- disintegrin family protein with thrombospondin motifs as an inflammation associated gene." Journal of Biological Chemistry **272**(1): 556-562.

Kunoh, H., N. Yamaoka, *et al.* (1988). "Preparation of the infection court by *Erysiphe graminis* I. Contact-mediated changes in morphology of the conidium surface." Experimental Mycology **12**: 325-335.

Kwon, Y. H. and L. Epstein (1993). "A 90-kDa glycoprotein associated with adhesion of *Nectria haematococca* macroconidia to substrata." Molecular Plant-Microbe Interactions **6**: 481-487.

Kwon, Y. H. and L. Epstein (1997). "Involvement of the 90 kDa glycoprotein in adhesion of *Nectria haematococca* macroconidia." Physiological and Molecular Plant Pathology **51**(5): 287-303.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**: 680-685.

Landfear, S. M. and M. Ignatushchenko (2001). "The flagellum and flagellar pocket of trypanosomatids." Molecular and Biochemical Parasitology **115**(1): 1-17.

Laval, V., M. Chabannes, *et al.* (1999). "A family of *Arabidopsis* plasma membrane receptors presenting animal b-integrin domains." Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology **1435**(1-2): 61-70.

Lawler, J. (1993). Thrombospondin. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 95-96.

Lawler, J., F. C. Chao, *et al.* (1982). "Evidence for calcium-sensitive structure in platelet thrombospondin. Isolation and partial characterization of thrombospondin in the presence of calcium." Journal of Biological Chemistry. **257**(20): 12257-12265.

Lawler, J. and R. O. Hynes (1986). "The structure of human thrombospondin, an adhesive glycoprotein with multiple calcium-binding sites and homologies with several different proteins." Journal of Cell Biology **103**: 1635-1648.

Lay, J. O., Jr. (2001). "MALDI-TOF mass spectrometry of bacteria." Mass Spectrometry Reviews **20**(4): 172-194.

Lehnen, L. P. and M. J. Powell (1989). "The role of kinetosome-associated organelles in the attachment of encysting secondary zoospores of *Saprolegnia ferax* to substrates." Protoplasma **149**: 163-174.

Letunic, I., L. Goodstadt, *et al.* (2002). "Recent improvements to the SMART domain-based sequence annotation resource." Nucleic Acids Research **30**(1): 242-244.

Liska, A. J. and A. Shevchenko (2003). "Combining mass spectrometry with database interrogation strategies in proteomics." TRAC-Trends in Analytical Chemistry **22**(5): 291-298.

Lord, E. M. (2003). "Adhesion and guidance in compatible pollination." Journal of Experimental Botany **54**(380): 47-54.

Lottspeich, F. (2000). Identification of proteins by amino acid sequencing. Proteome research: 2-dimensional gel electrophoresis and identification methods. R. Rabilloud. Heidelberg, Springer Verlag: 181-196.

Luu, D. T., D. Marty-Mazars, *et al.* (1999). "Pollen-stigma adhesion in *Brassica spp* involves SLG and SLR1 glycoproteins." Plant Cell **11**(2): 251-262.

Marqués-Calvo, M. S. (2002). "In vitro colonization of hydrophilic contact lenses by *Aspergillus niger*." Journal of Industrial Microbiology and Biotechnology **29**(1): 6-9.

Marshall, J. S., J. M. Wilkinson, *et al.* (2001). "Structure and expression of the genes encoding proteins resident in large peripheral vesicles of *Phytophthora cinnamomi* zoospores." Protoplasma **215**(1-4): 226-239.

Matthew, W. D. and P. H. Patterson (1983). "The production of a monoclonal antibody that blocks the action of a neurite outgrowth-promoting factor." Cold Spring Harbor Symposium of Quantitative Biology **48**(2): 625-631.

Matuschewski, K., A. C. Nunes, *et al.* (2002). "*Plasmodium* sporozoite invasion into insect and mammalian cells is directed by the same dual binding system." EMBO Journal **21**(7): 1597-1606.

May, K. J., S. C. Whisson, *et al.* (2002). "Inheritance and mapping of 11 avirulence genes in *Phytophthora sojae*." Fungal Genetics and Biology **37**(1): 1-12.

McFadden, G. I. and R. Wetherbee (1985). "Flagellar regeneration and associated scale deposition in *Pyramimonas gelidicola* (Prasinophyceae, Chlorophyta)." Protoplasma **128**(1): 31-37.

Meiniel, A. (2001). "SCO-spondin, a glycoprotein of the subcommissural organ/Reissner's fiber complex: evidence of a potent activity on neuronal development in primary cell cultures." Microscopy Research and Technique **52**(5): 484-495.

Meiniel, R., I. Creveaux, *et al.* (1995). "Specific transcripts analysed by in situ hybridization in the subcommissural organ of bovine embryos." Cell and Tissue Research **279**(1): 101-107.

Mellersh, D. G. and M. C. Heath (2001). "Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration." Plant Cell **13**(2): 413-424.

Mendgen, K. and H. Deising (1993). "Infection structures of fungal plant pathogens - a cytological and physiological evaluation." New Phytologist **124**: 193-213.

Mercure, E. W., H. Kunoh, *et al.* (1994b). "Adhesion of *Colletotrichum graminicola* conidia to corn leaves: a requirement for disease development." Physiological and Molecular Plant Pathology **45**: 407-420.

Mercure, E. W., H. Kunoh, *et al.* (1995). "Visualization of materials released from adhered, ungerminated conidia of *Colletotrichum graminicola*." Physiological and Molecular Plant Pathology **46**: 121-135.

Mercure, E. W., B. Leite, *et al.* (1994a). "Adhesion of ungerminated conidia of *Colletotrichum graminicola* to artificial hydrophobic surfaces." Physiological and Molecular Plant Pathology **45**: 421-440.

Miao, W. M., W. L. Seng, *et al.* (2001). "Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and - independent mechanisms." Cancer Research **61**(21): 7830-7839.

Mitchell, H. J. and A. R. Hardham (1999). "Characterisation of the water expulsion vacuole in *Phytophthora nicotianae* zoospores." Protoplasma **206**: 118-130.

Mitchell, H. J., K. A. Kovac, *et al.* (2002). "Characterisation of *Phytophthora nicotianae* zoospore and cyst membrane proteins." Mycological Research **106**(10): 1211-1223.

Mollet, J. C., S. Y. Park, *et al.* (2000). "A lily stylar pectin is necessary for pollen tube adhesion to an in vitro stylar matrix." Plant Cell **12**(9): 1737-1750.

Moloshok, T. D., G. M. E. Leinhos, *et al.* (1993). "The autogenic extracellular environment of *Uromyces appendiculatus* urediospore germlings." Mycologia **85**: 392-400.

Morris, P. F. and E. W. B. Ward (1992). "Chemoattraction of zoospores of the soybean pathogen, *Phytophthora sojae*, by isoflavones." Physiological and Molecular Plant Pathology **40**: 17-22.

Mosher, D. F. (1993). Vitronectin. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 101-102.

Mueller, W. E., H. C. Schroeder, *et al.* (2001). "Contribution of sponge genes to unravel the genome of the hypothetical ancestor of Metazoa (Urmetazoa)." Gene **276**(1-2): 161-173.

Mulvey, M. A. (2002). "Adhesion and entry of uropathogenic *Escherichia coli*." Cellular Microbiology **4**(5): 257-271.

Murdoch, L. J., I. Kobayashi, *et al.* (1998). "Production and characterisation of monoclonal antibodies to cell wall components of the flax rust fungus." European Journal of Plant Pathology **104**: 331-346.

Nakamura, S., G. Tanaka, *et al.* (1996). "Assembly and function of *Chlamydomonas* flagellar mastigonemes as probed with a monoclonal antibody." Journal of Cell Science **109**(1): 57-62.

Newhook, F. J. and F. D. Podger (1972). "The role of *Phytophthora cinnamomi* in Australian and New Zealand forests." Annual Review of Phytopathology **10**: 299-326.

Nicholson, R. L. (1996). Adhesion of fungal propagules. Histology, Ultrastructure and Molecular Cytology of Plant-Microorganism Interactions. M. Nicole and V. Gianinazzi-Pearson. Dordrecht, Kluwer Academic Publishers: 117-134.

Nicholson, R. L. and L. Epstein (1991). Adhesion of fungi to the plant surface. The Fungal Spore and Disease Initiation in Plants and Animals. G. T. Cole and H. C. Hoch. New York, Plenum Press: 3-23.

Nicholson, R. L., H. Kunoh, *et al.* (1993). "Initiation of the infection process by *Erysiphe graminis*: Conversion of the conidial surface from hydrophobicity to hydrophilicity and influence of the conidial exudate on the hydrophobicity of the barley leaf surface." Physiological and Molecular Plant Pathology **43**: 307-318.

Nielsen, H., S. Brunak, *et al.* (1999). "Machine learning approaches for the prediction of signal peptides and other protein sorting signals." Protein Engineering **12**(1): 3-9.

Nielsen, H., J. Engelbrecht, *et al.* (1997a). "Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites." Protein Engineering **10**(1): 1-6.

Nielsen, H., J. Engelbrecht, *et al.* (1997b). "A neural network method for identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites." International Journal of Neural Systems **8**(5-6): 581-599.

Nordhoff, E., V. Egelhofer, *et al.* (2001). "Large-gel two-dimensional electrophoresis-matrix assisted laser desorption/ionization-time of flight-mass spectrometry: an analytical challenge for studying complex protein mixtures." Electrophoresis **22**(14): 2844-2855.

Nualart, F., S. Hein, *et al.* (1998). "Partial sequencing of Reissner's fiber glycoprotein I (RF-Gly I)." Cell and Tissue Research **292**(2): 239-250.

Nyman, T. A. (2001). "The role of mass spectrometry in proteome studies." Biomolecular Engineering **18**(5 Special Issue SI): 221-227.

Oebrink, B. (1993). Cell adhesion and cell-cell contact proteins. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxfort University Press: 109-113.

Olmsted, J. B. (1981). "Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples." Journal of Biological Chemistry **256**(23): 11955-11957.

Osherov, N. and G. May (2000). "Conidial germination in *Aspergillus nidulans* requires RAS signaling and protein synthesis." Genetics **155**(2): 647-656.

Osherov, N. and G. S. May (2001). "The molecular mechanisms of conidial germination." FEMS Microbiology Letters **199**(2): 153-160.

Pain, N. A., R. J. O'Connell, *et al.* (1994). "Identification of glycoproteins specific to biotrophic intracellular hyphae formed in the *Colletotrichum lindemuthianum*-bean interaction." New Phytologist **127**: 233-242.

Pan, J. and W. J. Snell (2000). "Signal transduction during fertilization in the unicellular green alga, *Chlamydomonas*." Current Opinion in Microbiology **3**(6): 596-602.

Park, S. Y., G. Y. Jauh, *et al.* (2000). "A lipid transfer-like protein is necessary for lily pollen tube adhesion to an in vitro stylar matrix." Plant Cell **12**(1): 151-164.

Pascholati, S. F., H. Yoshioka, *et al.* (1992). "Preparation of the infection court by *Erysiphe graminis* f.sp. *hordei*: cutinase is a component of the conidial exudate." Physiological and Molecular Plant Pathology **41**: 53-59.

Patterson, D. J. (1989). Stramenoplies: chromophytes from a protistan perspective. The Chromophyte Algae: Problems and Perspectives. J. C. Green, B. S. C. Leadbeater and W. L. Diver. Oxford, Clarendon Press: 357-379.

Petersen, A. B. and S. Rosendahl (2000). "Phylogeny of the Peronosporomycetes (Oomycota) based on partial sequences of the large ribosomal subunit (LSU rDNA)." Mycological Research **104**(Part 11): 1295-1303.

Pieterse, C. M. J., P. Van West, *et al.* (1994). "Structure and genomic organization of the ipiB and ipiO gene clusters of Phytophthora infestans." Gene **138**: 67-77.

Quintans, J. and Z. S. Quan (1983). "Idiotype shifts caused by neonatal tolerance to phosphorylcholine." Journal of Immunology **130**(2): 590-595.

Qutob, D., P. T. Hraber, *et al.* (2000). "Comparative analysis of expressed sequences in *Phytophthora sojae*." Plant Physiology **123**(1): 243-254.

Randall, T. A. and H. S. Judelson (1999). "Construction of a bacterial artificial chromosome library of *Phytophthora infestans* and transformation of clones into *P. infestans*." Fungal Genetics and Biology **28**: 160-170.

Reichle, R. E. (1969). "Fine structure of *Phytophthora parasitica* zoospores." Mycologia **61**: 30-51.

Ridley, B. L., M. A. O'Neill, *et al.* (2001). "Pectins: structure, biosynthesis, and oligogalacturonide-related signaling." Phytochemistry **57**(6): 929-967.

Riethmueller, A., M. Weiss, *et al.* (1999). "Phylogenetic studies of Saprolegniomycetidae and related groups based on nuclear large subunit ribosomal DNA sequences." Canadian Journal of Botany **77**: 1790-1800.

Riggott, M. J. and W. D. Matthew (1996). "Generating monoclonal antibodies to neuronal antigens." Journal of Neuroscience Methods **68**(2): 235-245.

Ristaino, J. B. (2002). "Tracking historic migrations of the Irish potato famine pathogen, *Phytophthora infestans*." Microbes and Infection **4**(13): 1369-1377.

Robold, A. V. and A. R. Hardham (1998). "Production of species-specific monoclonal antibodies that react with surface components on zoospores and cysts of *Phytophthora nicotianae*." Canadian Journal of Microbiology **44**(12): 1161-1170.

Robson, K. J., S. Naitza, *et al.* (1997). "Cloning and expression of the thrombospondin related adhesive protein gene of *Plasmodium berghei*." Molecular and Biochemical Parasitology **84**(1): 1-12.

Rosenbaum, J. L. and G. B. Witman (2002). "Intraflagellar transport." Nature Reviews Molecular Cell Biology **3**(11): 813-825.

Rottem, S. (2003). "Interaction of mycoplasmas with host cells." Physiological Reviews **83**(2): 417-432.

Ruggeri, Z. M. and J. Ware (1993). Von Willebrand Factor. Guidebook to the extracellular matrix and adhesion proteins. T. E. Kreis and R. Vale. New York, Oxford University Press: 103-105.

Sambrook, J., E. F. Fritsch, *et al.* (1989). Molecular Cloning, A Laboratory Manual. New York, Cold Spring Harbor Laboratory Press.

Santoni, V., T. Rabilloud, *et al.* (1999). "Towards the recovery of hydrophobic proteins on two-dimensional electrophoresis gels." Electrophoresis **20**: 705-711.

Santoni, V., D. Rouqui, *et al.* (1998). "Use of a proteome strategy for tagging proteins present at the plasma membrane." Plant Journal **16**(5): 633-641.

Schultz, J., F. Milpetz, *et al.* (1998). "SMART, a simple modular architecture research tool: identification of signaling domains." Proceedings of the National Academy of Sciences of the United States of America **95**(11): 5857-5864.

Sela-Buurlage, M., L. Epstein, *et al.* (1991). "Adhesion of ungerminated *Colletotrichum musae* conidia." Physiological and Molecular Plant Pathology **39**: 345-352.

Silflow, C. D. and P. A. Lefebvre (2001). "Assembly and motility of eukaryotic cilia and flagella. Lessons from *Chlamydomonas reinhardtii*." Plant Physiology **127**(4): 1500-1507.

Sing, V. O. and S. Bartnicki-Garcia (1975a). "Adhesion of *Phytophthora palmivora* zoospores: detection and ultrastructural visualization of concanavalin A receptor sites appearing during encystment." Journal of Cell Science **19**: 11-20.

Sing, V. O. and S. Bartnicki-Garcia (1975b). "Adhesion of *Phytophthora palmivora* zoospores: electron microscopy of cell attachment and cyst wall fibril formation." Journal of Cell Science **18**: 123-132.

Skuce, P. J., G. F. J. Newlands, *et al.* (2001). "Cloning and characterisation of thrombospondin, a novel multidomain glycoprotein found in association with a host protective gut extract from *Haemonchus contortus*." Molecular and Biochemical Parasitology **117**(2): 241-244.

Snell, W. and W. Moore (1980). "Aggregation-dependent turnover of flagellar adhesion molecules in *Chlamydomonas* gametes." Journal of Cell Biology **84**(1): 203-210.

Sogin, M. L. and J. D. Silberman (1998). "Evolution of the protists and protistan parasites from the perspective of molecular systemics." International Journal for Parasitology **28**: 11-20.

Spano, F., L. Putignani, *et al.* (1998). "Molecular cloning and expression analysis of a *Cryptosporidium parvum* gene encoding a new member of the thrombospondin family." Molecular and Biochemical Parasitology **92**: 147-162.

Springer, T. A. (1980). Cell-Surface Differentiation in the Mouse. Monoclonal Antibodies: Hybridomas: A New Dimesion in Biological Analyses. R. H. Kennett and T. J. McKearn. New York, Plenum: 185-217.

Staab, J. F., S. D. Bradway, *et al.* (1999). "Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1." Science **283**(5407): 1535-1538.

Stanley, M. S., M. E. Callow, *et al.* (1999). "Monoclonal antibodies to adhesive cell coat glycoproteins secreted by zoospores of the green alga *Enteromorpha*." Planta **210**(1): 61-71.

Stensballe, A. and O. N. Jensen (2001). "Simplified sample preparation method for protein identification by matrix-assisted laser desorption/ionization mass spectrometry: in-gel digestion on the probe surface." Proteomics **1**(8): 955-966.

Sundstrom, P. (1999). "Adhesins in *Candida albicans*." Current Opinion in Microbiology **2**(4): 353-357.

Suryanarayana, K., D. D. Thomas, *et al.* (1985). "Calmodulin from the water mold *Achlya ambisexualis*: isolation and characterization." Cell Biology International Reports **9**(4): 389-400.

Svircev, A., R. Smith, *et al.* (1986). "Fungal fimbiae V. Protein A-gold immunocytochemical labeling of the fimbriae of *Ustilago violacea*." Experimental Mycology **10**: 19-27.

Talbot, N. J., M. J. Kershaw, *et al.* (1996). "MPG1 encodes a fungal hydrophobin involved in surface interactions during infection-related development of *Magnaporthe grisea*." Plant Cell **8**: 985-999.

Tang, K., D. Opalsky, *et al.* (2003). "Single nucleotide polymorphism analyses by MALDI-TOF MS." International Journal of Mass Spectrometry **226**(1): 37-54.

Terhune, B. T. and H. C. Hoch (1993). "Substrate hydrophobicity and adhesion of *Uromyces* urediospores and germlings." Experimental Mycology **17**: 241-252.

Tobias, J. W., T. E. Shrader, *et al.* (1991). "The N-end rule in bacteria." Science **254**(5036): 1374-1377.

Tomley, F. M., K. J. Billington, *et al.* (2001). "EtMIC4: a microneme protein from *Eimeria tenella* that contains tandem arrays of epidermal growth factor-like repeats and thrombospondin type-I repeats." International Journal for Parasitology **31**: 1303-1310.

Tomley, F. M. and D. S. Soldati (2001). "Mix and match modules: structure and function of microneme proteins in apicomplexan parasites." Trends in Parasitology **17**: 81-88.

Trottein, F., T. Triglia, *et al.* (1995). "Molecular cloning of a gene from *Plasmodium falciparum* that codes for a protein sharing motifs found in adhesive molecules from mammals and plasmodia." Molecular and Biochemical Parasitology **74**(2): 129-141.

Tsugita, A. (1987). "Developments in protein microsequencing." Advances in Biophysics **23**: 81-113.

Tyler, B. M. (2001). "Genetics and genomics of the oomycete host interface." Trends in Genetics **17**(11): 611-614.

Tzarfati-Majar, V., T. Burstyn-Cohen, *et al.* (2001). "F-spondin is a contact-repellent molecule for embryonic motor neurons." Proceedings of the National Academy of Sciences of the United States of America **98**(8): 4722-4727.

van der Lee, T., I. De Witte, *et al.* (1997). "AFLP linkage map of the oomycete *Phytophthora infestans*." Fungal Genetics and Biology **21**(3): 278-291.

van der Lee, T., A. Robold, *et al.* (2001). "Mapping of avirulence genes in *Phytophthora infestans* with amplified fragment length polymorphism markers selected by bulked segregant analysis." Genetics **157**(3): 949-956.

van West, P., S. Kamoun, *et al.* (1999a). "Internuclear gene silencing in *Phytophthora infestans*." Molecular Cell **3**(3): 339-348.

van West, P., B. M. Morris, *et al.* (2002). "Oomycete plant pathogens use electric fields to target roots." Molecular Plant-Microbe Interactions **15**(8): 790-798.

van West, P., B. Reid, *et al.* (1999b). "Green fluorescent protein (GFP) as a reporter gene for the plant pathogenic oomycete *Phytophthora palmivora*." FEMS Microbiology Letters **178**(1): 71-80.

Warburton, A. J. and J. W. Deacon (1998). "Transmembrane Ca^{2+} fluxes associated with zoospore encystment and cyst germination by the phytopathogen *Phytophthora parasitica*." Fungal Genetics and Biology **25**(1): 54-62.

Waugh, M., P. Hraber, *et al.* (2000). "The *Phytophthora* genome initiative database: informatics and analysis for distributed pathogenomic research." Nucleic Acids Research **28**(1): 87-90.

Wayne, R., M. Staves, *et al.* (1992). "The contribution of the extracellular matrix to gravisensing in characean cells." Journal of Cell Science **101**(3): 611-623.

Wedlich, D. (2002). "The polarising role of cell adhesion molecules in early development." Current Opinion in Cell Biology **14**(5): 563-568.

Weerakoon, N. D., J. K. Roberts, *et al.* (1998). "Isolation and characterization of the single b-tubulin gene in *Phytophthora cinnamomi*." Mycologia **90**(1): 85-95.

Weigle, W. O. (1973). "Immunological unresponsiveness." Advances in Immunology **16**: 61-122.

Whisson, S. C., A. Drenth, *et al.* (1995). "*Phytophthora sojae* avirulence genes, RAPD, and RFLP markers used to construct a detailed genetic linkage map." Molecular Plant-Microbe Interactions **8**(6): 988-995.

Whisson, S. C., T. van der Lee, *et al.* (2001). "Physical mapping across an avirulence locus of *Phytophthora infestans* using a highly representative, large-insert bacterial artificial chromosome library." Molecular Genetics and Genomics **266**(2): 289-295.

Wilkins, M. R., E. Gasteiger, *et al.* (1998). Protein identification and analysis tools in the ExPASy Server. 2-D Proteome Analysis Protocols. A. J. Link. New Jersey, Humana Press.

Wilkins, M. R., E. Gasteiger, *et al.* (1999). "High-throughput mass spectrometric discovery of protein post-translational modifications." Journal of Molecular Biology **289**(3): 645-657.

Wilkins, M. R., I. Lindskog, *et al.* (1997). "Detailed peptide characterization using PEPTIDEMASS - a World-Wide-Web-accessible tool." Electrophoresis **18**(3-4): 403-408.

Willats, W. G., L. McCartney, *et al.* (2001). "Pectin: cell biology and prospects for functional analysis." Plant Molecular Biology **47**(1-2): 9-27.

Witcombe, D. M., S. I. Belli, *et al.* (2003). "Molecular characterisation of EmTFP250: a novel member of the TRAP protein family in *Eimeria maxima*." International Journal for Parasitology **33**(7): 691-702.

Yarwood, A. (1989). Manual methods of protein sequencing. Protein Sequencing - a practical approach. J. B. C. Findlay and M. J. Geisow. Oxford ; New York, N.Y, IRL Press: 119-145.

Zentmyer, G. A. (1980). *Phytophthora cinnamomi* and the Diseases it Causes. St Paul, Minnesota, The American Phytopathological Society.

Zhang, W. and B. T. Cait (1998). ProFound-An expert system for protein identification. Proceedings of the 46th ASMS Conference on Mass Spectrometry and Allied Topics, Orlando, Florida.

Zhang, W. and B. T. Cait (1995). Protein identification by database searching: a Bayesian algorithm. 43rd ASMS Confrence on Mass Spectrometry and Allied Topics, Atlanta, Georgia.

Zhang, W. and B. T. Chait (2000). "ProFound: an expert system for protein identification using mass spectrometric peptide mapping information." Analytical Chemistry **72**(11): 2482-2489.

Zhu, J.-K., R. A. Bressan, *et al.* (1993). "Loss of arabinogalactan-proteins from the plasma membrane of NaCl-adapted tobacco cells." Planta **190**: 221-226.

Zinkl, G. M., B. I. Zwiebel, *et al.* (1999). "Pollen-stigma adhesion in *Arabidopsis*: a species-specific interaction mediated by lipophilic molecules in the pollen exine." Development **126**(23): 5431-5440.

Zwankhuizen, M. J., F. Govers, *et al.* (1998). "Development of potato late blight epidemics - disease foci, disease gradients, and infection sources." Phytopathology **88**(8): 754-763.

Appedix I: List of primers used to sequence *P. nicotianae* zoospore cDNA clone λZAPII-Pn14B7

Primer name	Primer sequence (5' to 3')
mgforw	GCA AAG GAT GAA AAC CAG GAG
mgforw2	TCA GCG ACA GCA ACT GGA AC
mgforw3	CAC CAG CCA ATT CAT CAT ATT C
mgforw4	ATC AAG CGA AGA TTG GCG AC
mgforw5	CGC AAG ATA ATG GCA ACG TCA G
mgrev	CAA GTT TAG CAG TAA TCG AGC C

Note: Primers ending with –forw are forward primers, primers ending with -rev are reverse primers.

Appendix II: BLASTX results after NCBI search with *P. nicotianae* zoospore cDNA clone λZAPII-Pn14B7

Part1

Accession no.	Expectation	Homology to sequence	Organism
gi|462439|sp|P33279|E2K1_RABIT, gi|109297|pir||A41284, gi|165003|gb|AAA31241.1|	4e-39	Eukaryotic translation initiation factor 2-alpha kinase 1	Rabbit
gi|13183720|gb|AAK15318.1|AF330008_1	9e-27	Eukaryotic translation initiation factor 2-alpha kinase 1	*Gallus gallus*
gi|6580979|gb|AAF18391.1|AF181071_1	2e-25	Heme-regulated initiation factor 2-alpha kinase	*Homo sapiens*
gi|11125768|ref|NP_055228.2|, gi|9963767|gb|AAG09683.1|AF183414_1, gi|13676376|gb|AAH06524.1|AAH06524	2e-25	Heme-regulated initiation factor 2-alpha kinase; Heme-sensitive initiation factor 2-alpha kinase; Hemin-sensitive initiation factor 2-alpha kinase; Heme-regulated initiation factor 2-alpha kinase	*Homo sapiens*
gi|20809345|gb|AAH28923.1|	2e-25	Eukaryotic translation initiation factor 2-alpha kinase 1	*Mus musculus*
gi|7243119|dbj|BAA92607.1|	2e-25	KIAA1369 protein	*Homo sapiens*
gi|14916582|sp|Q9BQI3|E2K1_HUMAN gi|13276633|emb|CAB66498.1|, gi|21740285|emb|CAD39152.1|	2e-25	Eukaryotic translation initiation factor 2-alpha kinase (Heme-regulated eukaryotic initiation factor eIF-2-alpha kinase; Heme-regulated inhibitor, HRI; Heme-controlled repressor, HCR; Hemin-sensitive initiation factor 2-alpha kinase); hypothetical protein	*Homo sapiens*
gi|7673102|gb|AAF66736.1|AF147094_1	2e-25	heme-regulated initiation factor 2-alpha kinase	*Homo sapiens*
gi|7839458|gb|AAF70289.1|AF255050_1	2e-25	heme-regulated eukaryotic initiation factor 2-alpha kinase; HRI	*Homo sapiens*

Appendix III: TBLASTX results with *P. nicotianae* zoospore cDNA clone λZAPII-Pn14B7 Part 1

Accession no.	Expectation	Homology to sequence	Organism
gi|13183719|gb|AF330008.1|AF330008	1e-39	Eukaryotic translation initiation factor 2-alpha kinase, mRNA	*Gallus gallus*
gi|9963766|gb|AF183414.1|AF183414	7e-38	hemin-sensitive initiation factor 2-alpha kinase, mRNA	*Homo sapiens*
gi|11125767|ref|NM_014413.2|	8e-38	heme-regulated initiation factor 2-alpha kinase (HRI), mRNA	*Homo sapiens*
gi|13676375|gb|BC006524.1|BC006524	8e-38	HRI, clone MGC:797 IMAGE:2989989, mRNA	*Homo sapiens*
gi|21740284|emb|AL834494.1|HSM805605	8e-38	mRNA; cDNA DKFZp761I021 (from clone DKFZp761I021)	*Homo sapiens*
gi|13276632|emb|AL136563.1|HSM801537	8e-38	mRNA; cDNA DKFZp761I011 (from clone DKFZp761I011)	*Homo sapiens*
gi|7839457|gb|AF255050.1|AF255050	9e-38	HRI, mRNA	*Homo sapiens*
gi|443688|gb|L27707.1|RATEIF2APK	9e-38	eukaryotic hemin-sensitive initiation factor 2-alpha kinase (eIF-2a), mRNA	Rat
gi|22761120|dbj|AK075192.1|	1e-37	cDNA FLJ90711 fis, clone PLACE1008282, highly similar to heme-regulated eukaryotic initiation factor eIF 2-alpha kinase (EC 2.7.1.-)	*Homo sapiens*
gi|14211558|gb|AY033898.1|	1e-37	HRI, mRNA	*Mus musculus*
gi|165002|gb|M69035.1|RABEIF2A	2e-37	eIF-2a, mRNA	*Oryctolagus cuniculus*
gi|7305014|ref|NM_013557.1|	3e-37	eukaryotic translation initiation factor 2-alpha kinase 1 (Eif2ak1), mRNA	*Mus musculus*
gi|3924930|gb|AF028808.1|AF028808	3e-37	hemin-sensitive initiation factor 2-alpha kinase mRNA	*Mus musculus*
gi|7243118|dbj|AB037790.1|	4e-37	KIAA1369 protein, mRNA, partial sequence	*Homo sapiens*
gi|6580978|gb|AF181071.1|AF181071	6e-37	HRI, mRNA	*Homo sapiens*
gi|6857780|ref|NM_010121.1|	7e-28	Eukaryotic translation initiation factor 2-alpha kinase 3 (Eif2ak3), mRNA	*Mus musculus*

Appendix IV: BLASTX results with *P. nicotianae* zoospore cDNA clone λZAPII-Pn14B7 Part 2

Accession no.	Expectation	Homology to sequence	Organism
gi|27370432|ref|NP_766516.1|, gi|26341040|dbj|BAC34182.1|	2e-31	Hypothetical protein C730036H08; unnamed protein product	*Mus musculus*
gi|26251712|gb|AAH40457.1|	5e-31	Calcium/calmodulin-dependent protein kinase II alpha subunit	*Homo sapiens*
gi|4758128|ref|NP_004725.1|, gi|2224679|dbj|BAA20824.1|	1e-30	Doublecortin and CaM kinase-like 1; Doublecortin-like kinase; KIAA0369	*Homo sapiens*
gi|6225242|sp|O15075|DCK1_HUMAN	1e-30	Serine/threonine-protein kinase DCAMKL1 (Doublecortin-like and CAM kinase-like 1)	*Homo sapiens*
gi|6716522|gb|AAF26675.1|AF155821_1	2e-30	protein serine/threonine kinase CPG16	*Mus musculus*
gi|17985955|ref|NP_445795.1|; gi|6225243|sp|O08875|DCK1_RAT gi|2062399|gb|AAC99476.1|	2e-30	Doublecortin and calcium/calmodulin-dependent protein kinase-1; Serine/threonine-protein kinase DCAMKL1 (Doublecortin-like and CaM kinase-like 1; Calcium/calmodulin dependent protein kinase type I-like CPG16); protein serine/threonine kinase CPG16	*Rattus norvegicus*
gi|25952114|ref|NP_057065.2|	2e-30	Calcium/calmodulin-dependent protein kinase IIA isoform 1; CaM-kinase II alpha chain; calcium/calmodulin-dependent protein kinase II alpha-B subunit; calcium/calmodulin-dependent protein kinase type II alpha chain; CaM kinase II alpha subunit; CaMK-II alpha subunit	*Homo sapiens*
gi|4589580|dbj|BAA76812.1|	2e-30	KIAA0968 protein	*Homo sapiens*

Appendix V: TBLASTX results with *P. nicotianae* zoospore cDNA clone λZAPII-Pn14B7 Part 2

Accession no.	Expectation	Homology to sequence	Organism
gi|26006150|dbj|AB093234.1|	6e-33	mKIAA0369 protein, mRNA	*Mus musculus*
gi|27370431|ref|NM_172928.1|	1e-31	hypothetical protein C730036H08 (C730036H08), mRNA	*Mus musculus*
gi|26341039|dbj|AK050312.1|	1e-31	adult male liver tumor cDNA, RIKEN full-length enriched library, clone:C730036H08 product: hypothetical Eukaryotic protein kinase containing protein	*Mus musculus*
gi|25144033|ref|NM_067170.2|	7e-31	Essential doublecortin and protein kinase domain containing protein, ZYGote defective : embryonic lethal ZYG-8 (90.0 kD), mRNA	*Caenorhabditis elegans*
gi|15485229|emb|AJ319870.1|CEL319870	7e-31	ZYG-8 protein, mRNA	*Caenorhabditis elegans*
gi|4758127|ref|NM_004734.1|	2e-30	doublecortin and CaM kinase-like 1 (DCAMKL1), mRNA	*Homo sapiens*
gi|2224678|dbj|AB002367.1|	2e-30	KIAA0369 gene, mRNA	*Homo sapiens*
gi|26251711|gb|BC040457.1|	2e-30	calcium/calmodulin-dependent protein kinase II alpha subunit, clone MGC:26106 IMAGE:4811948, mRNA	*Homo sapiens*
gi|9910163|ref|NM_019978.1|	2e-30	double cortin and calcium/calmodulin-dependent protein kinase-like 1 (DCAMKL1), mRNA	*Mus musculus*
gi|6716517|gb|AF155819.1|AF155819	2e-30	doublecortin-like kinase (Dclk) mRNA	*Mus musculus*
gi|2062398|gb|U78857.1|RNU78857	2e-30	serine/threonine kinase CPG16 (cpg16), mRNA	*Rattus norvegicus*

Appedix VI: List of primers used to sequence *P. cinnamomi* cDNA clone λgt11-Vsv

Primer name	Primer sequence (5' to 3')
λgt11-3	CCC GCG GCC GCG GTG GCG ACG ACT CCT GGA GCC CGT CAG
λgt11-4	CCC GCG GCC GCT TGA CAC CAG ACC AAC TGG TAA TGG TAG
RI prox	GCT CTA GAA CTA GTG GAT CCC
T3	ATT AAC CCT CAC TAA AGG GA
T7	TAA TAC GAC TCA CTC ACT ATA GGG
Forw-01	TCA CTC CGA CCT ACT TCA AC
Forw-02	TCA GCG GTC AAC AAC AAG
Forw-03	TAT TCC GCC TAC TCC TCC AC
Forw-04	ACG AAG CTC CGA ACA CGA AC
Forw-05	CTC AAG ACT GCA CTG TCA GC
Forw-06	GCA GAC GAA CTC GAA CTA TCA C
Forw-07	TCA ATT GCC AGG TCA GTA G
Forw-08	GTG ACA ACA GTA ACA ACG GCA G
Forw-09	CTC GCA ATG TGA CTC AAG CC
Forw-10	TGC CAG AAG TGC ATC GTG AG
Forw-11	CGT CCA TTG ACT GCG TTC TC
Forw-12	TGA CTG GAG CGA CTG TGA TG
Forw-13	CAG CTC TCA ACA GCA CCA TC
Forw-14	AGT CTC GCA CTC GTC AAG TC
Forw-15	ATC ACG CAG ATT GCT CTC TAC
Forw-16	CAG AAG GAC AAG TAC GGT GG
Forw-17	GAG GAC GCA TAC TCG CAA AG
Forw-18	GTC CTG CAT TGA GTG AGA CC
Rev-01	CGT AAC TAT CGC AGC CAG TC
Rev-02	CGA GTC TTC AGT CCC GAT GTA G
Rev-03	CTA CTC CAC TCA CTC ACC ACA C
Rev-04	CTT GCA TCC TCC ATA CAG GTC G
Rev-05	AAT GCT CCA GTC AGA CCA GG
Rev-06	GCA GTT GAT CGG AGG ACA AG
Rev-07	GTC TTG GCA TCA CAC ACC TG
Rev-08	TGA CGT AGT ATT CGT AAG GGC
Rev-09	TCA GCT CCA TTG TTG ACC G
Rev-10	AGA CCA GTC ACT CAC AAC GC
Rev-11	ATG ATG CTG GAC ACA GTG CC
Rev-12	CGT CCG ATG ATA GGC ATG AG

Note: Primers Forw-1 to Forw-18 are forward primers, primers Rev-01 to Rev-12 are reverse primers.

Appendix VII: BLASTX results after NCBI search with the nucleotide sequence of the *Vsv* gene

Accession no.	Expectation	Homology to sequence	Organism
gi|28195400|ref|NP_777131.1|, gi|20145484|emb|CAC94914.1|	3-52	SCO-spondin	*Bos taurus*
gi|28524247|ref|XP_162416.2|	5e-49	SCO-spondin	*Mus musculus*
gi|27527438|emb|CAD42654.1|	5e-49	SCO-spondin	*Mus musculus*
gi|20521710|dbj|BAA76804.2|	5e-43	KIAA0960 protein	*Homo sapiens*
gi|12697903|dbj|BAB21770.1|	1e-41	KIAA1679 protein	*Homo sapiens*
gi|19032247|emb|CAD24309.1|	4e-34	putative coagulation serine protease	*Ciona intestinalis*
gi|17550024|ref|NP_510116.1|; gi|7494529|pir||T18856	9e-32	ADAMTS family ADT-1 (adt-1); angiogenesis inhibitor homolog	*Caenorhabditis elegans*
gi|21901950|dbj|BAC05514.1|, gi|23953896|emb|CAA90293.2|, gi|24817534|emb|CAA90302.2|	9e-32	ADT-1	*Caenorhabditis elegans*
gi|18544471|ref|XP_046570.3|	6e-31	similar to KIAA1679 protein	*Homo sapiens*
gi|27369658|ref|NP_766073.1|; gi|26342140|dbj|BAC34732.1|	3e-29	RIKEN cDNA D130067I03; hypothetical protein B930082A18; unnamed protein product	*Mus musculus*
gi|5326919|emb|CAB46239.1|	2e-28	SCO-spondin	*Bos taurus*
gi|17939568|gb|AAH19344.1|AAH19344; gi|21620031|gb|AAH33125.1|	7e-27	Unknown (protein for IMAGE:3530946); Unknown (protein for IMAGE:3028838)	*Homo sapiens*
gi|27710286|ref|XP_231721.1|	3e-26	similar to SCO-spondin	*Rattus norvegicus*
gi|5281383|gb|AAD41495.1|AF149302_1	8e-25	F-spondin precursor	*Gallus gallus*
gi|27478441|ref|XP_166543.3|	2e-23	similar to KIAA0960 protein	*Homo sapiens*
gi|27807443|ref|NP_777168.1|; gi|11320818|dbj|BAB18460.1|	8e-23	spondin 1, (f-spondin) extracellular matrix protein; VSGP/F-spondin	*Bos taurus*
gi|2388718|gb|AAC48311.1|	2e-22	thrombospondin related adhesive protein	*Cryptosporidium parvum*

Appendix VIII: TBLASTX results after NCBI search with the nucleotide sequence of the *Vsv* gene

Accession no.	Expectation	Homology to sequence	Organism
gi|23575283|dbj|AK112308.1|	2e-57	cDNA clone:ciad015e10	*Ciona intestinalis*
gi|23577510|dbj|AK113409.1|	1e-28	cDNA clone:ciad050c13	*Ciona intestinalis*
gi|20521709|dbj|AB023177.2|	9e-25	mRNA for KIAA0960 protein	*Homo sapiens*
gi|12697902|dbj|AB051466.1|	3e-23	mRNA for KIAA1679 protein	*Homo sapiens*
gi|5281382|gb|AF149302.1|AF149302	3e-17	F-spondin precursor	*Gallus gallus*
gi|204176|gb|M88469.1|RATFSAA	1e-16	F-spondin mRNA	*Rattus norvegicus*
gi|7022928|dbj|AK001584.1|	6e-25	cDNA FLJ10722 fis clone NT2RP3001140	*Homo sapiens*
gi|11320819|dbj|AB051390.1|	1e-24	mRNA for VSGP/F-spondin	*Homo sapiens*
gi|20521645|dbj|AB018305.2|	7e-24	mRNA for KIAA0762 protein	*Homo sapiens*
gi|11320817|dbj|AB051389.1|	3e-23	mRNA for VSGP/F-spondin	*Bos taurus*
gi|26101501|dbj|AK083798.1|	4e-23	12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130011L22	*Mus musculus*
gi|26342139|dbj|AK051714.1|	1e-22	12 days embryo spinal ganglion cDNA, RIKEN full-length enriched library, clone:D130067I03	*Mus musculus*
gi|2529224|dbj|AB006086.1|	2e-22	mRNA for F-spondin 1	*Danio rerio*
gi|409244|gb|L09123.1|XELTHSPOND	1e-20	mRNA for F-spondin	Frog
gi|22760485|dbj|AK074803.1|	1e-20	cDNA FLJ90322 fis, clone NT2RP2001755	*Homo sapiens*
gi|2529226|dbj|AB006087.1|	2e-16	mRNA for F-spondin2	*Danio rerio*
gi|3328303|gb|AF073838.1|AF073838	8e-12	thrombospondin related adhesive protein (TRAP-C3) gene	*Cryptosporidium parvum*
gi|509280|emb|X77587.1|CPMICRP	1e-17	TRAP-C1 gene for thrombospondin related adhesive protein (putative)	*Cryptosporidium parvum*

List of publications

Robold and Hardham (1998). Production of species-specific monoclonal antibodies that react with surface components on zoospores and cysts of *Phytophthora nicotianae*. Canadian Journal of Microbiology 44 (12): 1161-1170.

van der Lee, Robold, et al. (2001). Mapping of avirulence genes in *Phytophthora infestans* with amplified fragment length polymorphism markers selected by bulked segregant analysis. Genetics 157 (3): 949-956.

van der Lee, Testa, et al. (accepted for publication). High density genetic linkage maps of *Phytophthora infestans* reveal trisomic progeny and chromosomal rearrangements.

Robold and Hardham (accepted for publication). Production of monoclonal antibodies against peripheral vesicle proteins in zoospores of *Phytophthora nicotianae*.